

A STUDY OF ALDOSTERONE-BINDING COMPONENTS  
OF RAT KIDNEY

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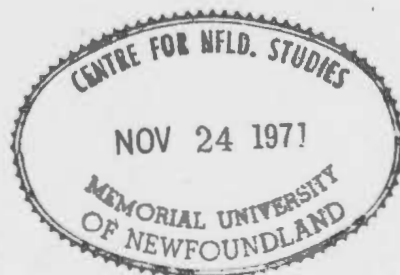
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A STUDY OF ALDOSTERONE-BINDING COMPONENTS  
OF RAT KIDNEY

A thesis

by

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Submitted in partial fulfillment of  
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the degree of Master of Science

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## ABSTRACT

We have confirmed that aldosterone binds to macromolecular receptors in the nuclear and cytosol fractions of rat kidney in vivo and in vitro. A lesser degree of aldosterone binding in vitro is apparent in fractions of heart, liver, and blood; no differences in binding to kidney components were apparent in male and female animals.

Binding to kidney components appears unstable even at 0°; some stabilization is offered by the presence of aldosterone or 20% glycerol. Aldosterone-macromolecular complexes formed in vivo are susceptible to dissociation by temperature increases above 0° and by pH extremes, but not by hyaluronidase. In vitro studies show that p-HMB inhibits complex formation and that aldosterone does not protect against such inhibition.

The binding of aldosterone to kidney components is greater in adrenalectomized animals than in unoperated controls and this increase in binding is apparent at 1 - 2 hours after surgery.

Attempts at purification of aldosterone-binding components by Sephadex chromatography and on sucrose density gradients were unsuccessful.

Kidney appears to contain at least two components which bind aldosterone:

(ii)

- (a) a relatively stable, specific component with a low affinity for aldosterone, and
- (b) an unstable, less specific component whose affinity for aldosterone has not been determined.

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## ABBREVIATIONS

Tris	-	tris(hydroxymethyl)aminomethane
p-HMB	-	para-hydroxymercuribenzoate
DNA	-	deoxyribonucleic acid
RNA	-	ribonucleic acid
fmole	-	femtomole ( $10^{-15}$ mole)
DOC	-	deoxycorticosterone
N.E.	-	nuclear extract
dpm	-	disintegrations per minute
c-AMP	-	adenosine 3':5'-cyclic monophosphate
ATP	-	adenosine triphosphate
B.S.A.	-	bovine serum albumin
nM	-	nanometer ( $10^{-9}$ meter)
O.D.	-	optical density
RNase	-	ribonuclease
DNase	-	deoxyribonuclease
p-CMB	-	para-chloromercuribenzoate
Na-K-ATPase	-	sodium, potassium activated adenosine triphosphatase

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## INTRODUCTION

### A. General

Hormones have been defined classically as chemical messengers, secreted in trace amounts by living cells, which regulate and initiate reactions at sites remote from their sites of production within an organism. Many hormones have been identified and classified but the mode of action of any hormone has not as yet been completely elucidated. In 1963 Karlson reviewed the concepts held on the mechanism of action of hormones and proposed a new explanation of the mode of hormone action<sup>1</sup> which was based on the outcome of an experimental study of chromosomal activity<sup>2</sup>.

The possible sites through which hormones may exert their effect in the cell are outlined below.

- 1) Hormones may control enzyme activity. This theory originated in 1941 with Green's hypothesis that substances functioning in trace amounts in biological systems must do so by influencing enzyme systems<sup>3</sup>.
- 2) Hormones may control cell permeability. Levine and Goldstein studied the increase in cell permeability to sugars brought about by insulin<sup>4</sup>, and Sharp and Leaf hold the view that the primary action of aldosterone is on the cell membrane<sup>5</sup>. They suggest that the hormone stimulates production of a protein which acts like a permease in facilitating entry of sodium through the mucosal surface of the tissue.

3) Hormones may control gene activity. This theory was propounded by Karlson as a result of his experimental study of chromosomal activity in certain insects<sup>2</sup>. Close inspection of the giant chromosomes of the salivary glands of certain insects such as the midge *Chironomus* shows that there are some sites where the structure becomes puffed up. This is probably caused by the unfolding of segments of DNA during the development of the organism and the resulting "puffs" have been identified as sites of RNA synthesis. Karlson showed that injection of the hormone ecdysone into the insect induced the puffing phenomenon and that the induction process occurred as a direct effect of ecdysone administration. He suggested that hormones act as inducers, which by combining with appropriate repressors, control m-RNA synthesis and thereby regulate enzyme synthesis.

This suggestion by Karlson aroused much excitement at the time but in the light of recent developments, Tata has suggested that a revision of Karlson's original idea is required<sup>6,7</sup>. The developments may be summarized as follows:

- (i) In 1965 Girard and co-workers presented evidence that m-RNA synthesized in the nucleus is transferred to the cytoplasm only when attached to the smaller ribosomal component<sup>8</sup>.
- (ii) However, Spirin and his group have offered evidence for the existence of m-RNA-containing nucleoproteins or

informosomes<sup>9</sup>. This component sediments immediately after 80 S ribosomes on sucrose density gradients and Spirin suggests that informosomes may be a form of m-RNA transfer from nucleus to cytoplasm and then into the polyribosomes.

(iii) Evidence has now appeared that various hormones may exert their effect by controlling the translational activity of cytoplasmic polyribosomes<sup>10,11</sup>. Tomkins et al have shown that the dexamethasone induction of tyrosine transaminase in hepatoma tissue culture cells is inhibited by cycloheximide or puromycin<sup>11</sup>. Although actinomycin D also inhibits enzyme induction when given at the same time as the inducer, Tomkins offers evidence that the inducer is required for the translation of the tyrosine transaminase messenger. He suggests that the actinomycin D effect may be a secondary result of the action of inducer on translation, or alternately, that there is a direct action of the hormones on the transcription process as well.

Hence the evidence seems to suggest that hormones may control cellular processes by influencing genetic expression. It is possible that hormones may intervene simultaneously at two or more points in the processes of transcription and translation of the genetic message.



## B. Early Events in the Action of Hormones

### 1) Effects on RNA synthesis -

It has been shown that the action of estradiol<sup>12</sup>, testosterone<sup>13</sup>, hydrocortisone<sup>14</sup>, growth hormone<sup>15</sup>, cortisone<sup>16</sup>, and aldosterone<sup>17-20</sup> is blocked by inhibitors of RNA synthesis. Hence it appears that the synthesis of RNA is an early event in the mechanism of action of many hormones.

### 2) Effects on protein synthesis -

Protein synthesis appears to be an early event in the mechanism of action of hormones since inhibitors of protein synthesis block the action of many hormones<sup>12-20</sup>.

O'Malley and co-workers have recently presented evidence that a single dose of progesterone to estrogen-stimulated chicks results in the induction of synthesis of a specific oviduct protein, avidin<sup>21</sup>.

### 3) Changes in chromatin activity -

Dahmus and Bonner have obtained evidence that administration of hydrocortisone to adrenalectomized rats increases the template efficiency of liver chromatin preparations<sup>22</sup>. More recently, Morgan and Bonner have shown that there is a 28% increase in the template activity of liver chromatin of insulin-treated diabetic rats compared to that of liver chromatin of untreated diabetic controls<sup>23</sup>.

Cherry and co-workers have shown, however, that in

soybean hypocotyl tissue the synthetic auxin, 2,4-dichlorophenoxyacetic acid, acts at the level of chromatin-bound RNA polymerase with relatively little change in template activity<sup>24</sup>.

#### 4) Changes in histones -

Bonner and co-workers have described a number of experiments which suggest that histones might be the factors that control gene activity<sup>25,26</sup>. Their regulatory function might be mediated by changes in rates of synthesis or degradation of specific histones or by modifications in histone structure which may alter the specificity of DNA - histone binding. The processes of acetylation, methylation, phosphorylation, and thiolation have been suggested as possible ways of altering histone structure and thereby influencing repressor activity<sup>27-31</sup>.

Allfrey and co-workers have presented evidence that histone acetylation is increased in the livers of animals within a short time after injection of hydrocortisone<sup>32</sup>. Other workers found that hydrocortisone stimulated the incorporation in vivo of [<sup>32</sup>P] orthophosphate into both the lysine-rich and arginine-rich histone fractions of liver nuclei of adrenalectomized rats<sup>33</sup>.

#### 5) Involvement of c-AMP -

An early event which follows administration of some hormones is an increase in the level of c-AMP in a particular target tissue<sup>34</sup> and this cyclic nucleotide has been suggested

to act as a mediator in the action of some hormones<sup>34,35</sup>.

#### 6) Hormone receptors -

The binding of hormone to receptors in target tissues appears to be an early event in the action of some hormones. Estradiol<sup>36</sup>, testosterone<sup>37,38</sup>, aldosterone<sup>39,40</sup>, progesterone<sup>41-43</sup>, and c-AMP<sup>44,45</sup> bind to receptor proteins in target tissues. Some of these receptors will be discussed in detail in the next section.

### C. Hormone Receptors and Target Tissues

#### 1) The receptor hypothesis -

The classical receptor hypothesis of hormone action suggests that target tissues contain molecules which specifically interact with hormones, and that as a result of such interactions, the receptor molecule's biological function is modified. This would explain the importance of stereochemical factors. There was little experimental evidence to support this hypothesis until Jensen and Jacobson studied the uptake of <sup>3</sup>H-estradiol-17 $\beta$  by various tissues of the immature rat<sup>46</sup>. They found that when physiological doses of estrogen were injected subcutaneously, tissues showing dramatic growth responses to the hormone also showed higher uptake and prolonged retention of the hormone. This finding renewed interest in the receptor hypothesis since it demonstrated that target tissues did differ from other tissues in their affinity for estrogen.

Gorski and co-workers have confirmed that rat uterus contains receptors for estrogens<sup>36,47-49</sup> and recent evidence suggests that the receptors are proteins<sup>48,49</sup>. It has been suggested that the estradiol receptor serves to concentrate the hormone from the peripheral blood<sup>47-51</sup>, and more recently, other workers have proposed that the receptor proteins function in the transfer of estrogen from the cytoplasm to the nucleus<sup>36,52</sup>.

Receptors for other steroid hormones in target tissues have been reported by various groups<sup>37-43,53</sup>. Two groups of workers have reported the presence of androgen receptors in male accessory sex tissue<sup>37,38</sup>; Edelman's group described partial purification of aldosterone receptors in rat kidney<sup>39,40</sup>; O'Malley has described progesterone receptors in chick oviduct tissue<sup>41-43</sup>; and Beato and co-workers have studied cortisol-binding macromolecules in rat liver<sup>53</sup>.

It is interesting to note that the tissues which respond to the administration of a particular hormone, i.e. the "target" tissues, contain the highest concentrations of receptor moieties<sup>43,54</sup> but that some "non-target" tissues may contain small amounts of receptor<sup>54</sup>. Hence it is possible that the ability of a given tissue to respond to a specific hormone may be directly proportional to the concentration of specific hormone receptor in the tissue; and, in addition, that the target tissue concept may require modification when more sensitive methods of measuring hormone responses become available.

2) Estrogen-binding components of target tissues -

The demonstration of preferential uptake and retention of  $17\beta$ -estradiol by target tissues of the rat<sup>46,50</sup> initiated much research on the interaction of this hormone with receptors in target tissues<sup>36,47-52,57-64</sup>. The binding of hormone to receptor appears to be a primary step in the mechanism of action of estradiol since it is not affected by such inhibitors of early estrogen response as puromycin or actinomycin D but is affected by estrogen analogues or inhibitors of estrogen action<sup>56</sup>.

A study of the distribution of radioactivity in sub-cellular fractions of the uterus after in vivo injection of  $^3\text{H}$ -estradiol into immature rats was made by Noteboom and Gorski<sup>47</sup>. It was revealed that 50% of the radioactivity was in the nuclear fraction while cytosol contained 30%. Similar results were obtained by Talwar et al<sup>51</sup> and by Puca and Bresciani<sup>57</sup> using ovariectomized rats. The majority of the steroid seems to be present as unchanged estradiol up to 6 hr after subcutaneous administration of the radioactive hormone<sup>46,55,56</sup>.

King et al showed that after disruption of the nuclei by ultrasonification, approximately 50% of the radioactivity in nuclei from mammary tumors, anterior pituitary, kidney, and uterus was associated with the chromatin fraction<sup>59</sup>. It was demonstrated that incubation of labelled nuclear fraction

with either RNase or DNase did not release radioactivity from the chromatin, whereas radioactivity was released on incubation with trypsin<sup>47,57,60</sup>. This suggested that the estradiol-binding component was a protein and analyses of 0.3 M KCl extracts of uterine nuclei on sucrose density gradients revealed that the major radioactivity peak sedimented in the 5S range<sup>36</sup>,

Puca and Bresciani were able to demonstrate in vitro binding of estradiol to 0.3 M KCl extracts of uterine nuclei<sup>57</sup>. They found that the amount of estrogen bound by the extract increased rapidly with estrogen concentration until a saturation point was reached and then the binding increased slowly. It was suggested from these results that there were two binding sites for estrogen, one with a high affinity and low capacity and the other with a low affinity.

The estradiol binding in cytosol was investigated by Toft and Gorski<sup>48</sup>. They showed that after in vivo injection of low doses of <sup>3</sup>H-estradiol to rats, a single peak of radioactivity was obtained and this sedimented at 9.5 S in sucrose density gradients. In rats receiving higher doses an additional broad peak was observed in the 4-6 S region. The cytosol binding component was shown to be a protein since proteolytic enzymes dissociated the complex but DNase and RNase had no effect.

The binding of estradiol to cytoplasmic receptors in cell free systems has been demonstrated by Toft et al<sup>49</sup> and by Talwar

et al<sup>61</sup>. A value of  $10^{-9}$  M was calculated for the concentration of cytoplasmic binding sites for estradiol in the uterus of the ovariectomized rats. Heat treatment ( $60^{\circ}$ , 4 min), detergents (0.5% sodium dodecyl sulfate) and sulfhydryl reagents (2 mM p-CMB or 1 mM ethylmaleimide) almost completely abolished binding. Jensen et al had shown previously that labelled estradiol, incorporated into rat uterus after injection of hormone in vivo, or by exposure to the hormone in vitro, could be released by adding sulfhydryl reagents to the washing medium<sup>62</sup>. Hence it appears that sulfhydryl reagents may be directly involved in the estrogen-receptor interaction.

Shyamala and Gorski incubated intact uteri with estradiol at  $0^{\circ}$  and found that most of the estrogen retained by the uterus was present in the cytosol as an 8 S complex<sup>36</sup>. If the uteri were transferred to estrogen-free medium at  $37^{\circ}$  the estrogen was now found in the nucleus in association with a 5 S protein. Now if the uteri were returned to an estrogen-free medium at  $0^{\circ}$ , the estrogen did not return to the cytosol, and the ability of the cytosol to bind estrogen was lost. These observations suggested that the 5 S estradiol-protein complex of the nucleus is somehow derived from the 8 S estradiol-protein complex of the cytoplasm and led to the proposal of a two-step mechanism for the interaction of estradiol with rat uterus<sup>36,52</sup>.

The most recent work by Giannopoulos and Gorski suggests that the native state of estrogen binding protein has a single estrogen binding site and that the different estrogen binding proteins observed on sucrose density gradients (8S, 4S, 5S, 6S) all contain a common estrogen binding site<sup>63,64</sup>. They may represent different states of aggregation of one or more types of protein subunits.

3) Aldosterone-binding components of target tissues -

Aldosterone has been shown to influence the transport of sodium across cell membranes in mammalian kidneys and intestines<sup>65</sup>, sweat glands<sup>66</sup>, salivary glands<sup>67</sup>, and in certain anuran epithelia such as the toad bladder<sup>68</sup> by a process which involves DNA-dependent RNA synthesis. However, in order to determine the mechanism of action of mineralocorticoids, it is necessary to identify and characterize the first step in the process, the interaction of the steroid with its receptors in target tissues.

The binding of hormone to receptor is probably non-covalent since the native steroid is extracted readily into  $\text{CH}_2\text{Cl}_2$  from kidney fractions and from prelabelled toad bladder<sup>17,70,71</sup>.

Edelman and co-workers have recently isolated, partially purified, and characterized aldosterone-macromolecular complexes from rat kidney nuclear and cytosol fractions after subcutaneous injection of  $^3\text{H}$ -aldosterone<sup>40</sup>. The complexes are



dissociated by proteolytic enzymes but not by nucleases, are specific for mineralocorticoids, and are susceptible to destruction by sulfhydryl reagents. Preliminary studies suggested that aldosterone-protein complexes could be formed in vitro and that these complexes might be identical to those formed in vivo.

More recently, it has been demonstrated that aldosterone binds to chromatin in the nucleus and glycerol density gradient centrifugation revealed that the primary binding unit was a 4 S non-histone chromosomal protein<sup>72</sup>.

Aldosterone binding components are unstable and tend to dissociate with time after isolation. It has been shown that some stabilization is afforded by the presence of glycerol or aldosterone<sup>73</sup>.

Heterogeneity of aldosterone binding components has been suggested from the kinetics of uptake of <sup>3</sup>H-aldosterone by rat kidney nuclei and in the binding of <sup>3</sup>H-aldosterone to the intact toad bladder<sup>70,71</sup>.

A comparison of the properties of mineralocorticoid-binding proteins with those of estrogen-binding proteins reveals striking similarities. Mineralocorticoid and estrogen action are probably both initiated by a similar process which involves the formation of specific steroid-receptor complexes and the elucidation of mechanism of action of steroid hormones will probably await the further characterization of the first step in the process, the interaction of the hormone with its receptor protein.

#### D. Adrenal Cortex-Kidney Interactions

Adrenocorticoids are steroid hormones produced by the adrenal cortex. They influence glucose and salt metabolism in various tissues and accumulated evidence has established that kidney is one target tissue for the action of adrenocorticoids.

Katz and Epstein have reported that Na-K-ATPase activity increased when sodium reabsorption was increased by feeding rats a high-protein diet but that Na-K-ATPase activity was reduced when sodium transport was diminished after bilateral adrenalectomy<sup>74,75</sup>. Manitius et al have shown that glucocorticoid stimulation of Na-K-ATPase activity is caused by an increase in the quantity of plasma membrane per cell rather than an increase in the activity of enzyme per unit of membrane<sup>76</sup>.

Jørgenson has demonstrated that the reduction in Na-K-ATPase activity during developing adrenal insufficiency corresponds to the rate of change in concentration of sodium and potassium in plasma<sup>77</sup>. He found, however, that a high sodium intake could partly prevent the reduction in enzymatic activity and suggested that Na-K-ATPase is at least partly influenced by the sodium concentrations in plasma.

Stolte et al examined the mechanism of inhibition of water diuresis in adrenal insufficiency by the direct method of perfusing single tubules of rat kidney<sup>78</sup>. The authors suggest that glucocorticoids act directly on distal

tubules to decrease their permeability to water.

The work of Kirsten et al has demonstrated that the most potent mineralocorticoid, aldosterone, stimulates the activities of some enzymes of the TCA cycle when added to the media of isolated toad bladders<sup>79</sup>. However, the effect of aldosterone on enzyme activities and on sodium transport were considered to be independent actions of the hormone since the increase in enzyme activity was not dependent on the presence of sodium in the bathing medium and physiological concentrations of aldosterone were not employed.

Liu, Liew, and Gornall, however, have reported recently that specific activities of some mitochondrial enzymes of rat kidney are lowered following adrenalectomy and that administration of aldosterone restores the enzyme activities to control values<sup>80</sup>. These findings support Edelman's view that aldosterone acts via a metabolic pathway<sup>17</sup>.

Some experimental evidence has implicated the membrane as the site of action of adrenocortical hormones<sup>81,82</sup>. Total phospholipids were reduced in kidney after adrenalectomy<sup>81</sup>. This was due mainly to a reduction in the content of phosphatidylcholine. DeVenuto and Lange have shown that kidney microsomes of adrenalectomized rats demonstrated a higher <sup>14</sup>C-leucine incorporation activity than those of kidneys from normal animals at times ranging from 3 hours to 31 days after adrenalectomy<sup>82</sup>.

Davis et al have suggested that sodium depletion in dogs stimulates aldosterone production in the adrenal gland by accelerating the conversion of corticosterone to aldosterone and probably of cholesterol to pregnenolone<sup>83</sup>. Marusic and Mulrow have demonstrated an accelerated rate of conversion of corticosterone to aldosterone by sodium depletion in rats<sup>84</sup>.

The independent work of Laragh and of Genest demonstrated the ability of angiotensin II to stimulate the secretion of aldosterone from the adrenal gland<sup>85,86</sup>. Angiotensin II is an octapeptide formed by a converting enzyme acting on angiotensin I which in turn is formed by the action of a kidney enzyme, renin, on a plasma  $\alpha$ -globulin.

It seems that kidney contains factors which influence the rate of secretion of adrenocorticoids and that metabolic processes in the kidney may be regulated by adrenocorticoids in a process which involves, either directly or indirectly, control of enzyme activities, m-RNA and protein synthesis, and synthesis of components of the cell membrane. The feedback mechanisms operating between kidney and adrenal in normal mammalian systems render the two organs somewhat interdependent.

## MATERIALS AND METHODS

All non-radioactive hormones used in this work, with the exception of aldosterone, were purchased from Sigma Chemical Co., St. Louis, Mo. Aldosterone was purchased from Calbiochem, Los Angeles, California. All other chemicals, unless otherwise specified, were obtained from J.T. Baker Chemical Co., Phillipsburg, N.J., Sigma Chemical Co., or Matheson, Coleman and Bell Manufacturing Chemists, Norwood, Ohio. All chemicals were of the analytical grade purity.

Radioactive  $^3\text{H}$ -aldosterone, specific activity 32 curies per millimole and radiochemical purity greater than 95% as determined by descending paper chromatography, was purchased from New England Nuclear, Boston, Mass.

$^3\text{H}$ -water, specific activity 1 millicurie per gram, was purchased from New England Nuclear.

Sephadex G-50, G-75, and G-200, and Sephadex columns were purchased from Pharmacia, Uppsala, Sweden.

### Preparation of hormone solutions

$10^{-3}\text{M}$  hormone solutions were prepared in 95% ethanol and diluted to  $10^{-6}\text{M}$  with solution A. All hormone solutions were prepared immediately before use.

#### Preparation of other reagents

The following reagents were used throughout this work and will be referred to as solutions "A" and "B".

Solution A - (Tris- $\text{CaCl}_2$  buffer, pH 7.4)

0.1 M Tris, 3 mM  $\text{CaCl}_2$ , adjusted to pH 7.4 with concentrated HCl.

Solution B - (Scintillation Fluid)<sup>71</sup>

10 gm 2,5 diphenyloxazole (PPO)

0.5 gm 1,4-bis-2-(5-phenyloxazolyl) benzene (POPOP)

80 gm naphthalene

428 ml ethylene glycol monomethyl ether

428 ml p-dioxane

143 ml xylene

#### Adrenalectomy and administration of $^3\text{H}$ -aldosterone

Female Sprague-Dawley rats, weighing 75-150 gm, were used in these experiments unless otherwise specified. Animals were obtained from Canadian Breeding Laboratories, St. Constant, Quebec, or from the Memorial University of Newfoundland Medical School, St. John's, Newfoundland. Bilateral adrenalectomy was performed by the dorsal route and adrenalectomized animals were sustained on 1% sodium chloride and Purina Laboratory Chow ad libium. The rats were sacrificed 6-12 days after the operation unless otherwise specified.

In in vivo experiments  $5 \times 10^{-10}$  mole of  $^3\text{H}$ -aldosterone in 1.0 ml of 0.9% sodium chloride was injected intraperitoneally 45 min before sacrifice. The kidneys were removed, nuclear extracts and cytosol fractions were obtained, and the assays for radioactivity and protein were carried out immediately, unless otherwise stated.

In in vitro experiments  $^3\text{H}$ -aldosterone was added either before homogenization or after the fractions were isolated. Additions to isolated fractions were made immediately after isolation. Where addition of hormone was made prior to isolation of fractions, the following procedure was employed routinely.  $^3\text{H}$ -aldosterone was added to the 0.25 M sucrose homogenization medium before the addition of kidney.

$^3\text{H}$ -aldosterone was added also to the solution "A" nuclear extraction medium before re-homogenizing the nuclear pellet. Unless otherwise indicated, fractions were incubated 2.5 hr at  $0 - 4^\circ$  before assay. Aldosterone concentrations were  $5 \times 10^{-10}$  M unless otherwise indicated.

Where  $^3\text{H}$ -aldosterone was present in the isolation media, the total time of contact of  $^3\text{H}$ -aldosterone with the nuclear binding components was 3.5 hr (1 hr isolation time plus 2.5 hr incubation time). Similarly, the total time of contact with cytoplasmic components was 4 hr (1.5 hr isolation time plus 2.5 hr incubation time).

In the kinetics experiments concentrations of aldosterone above  $10^{-8}$  M were obtained by diluting  $^3\text{H}$ -aldosterone with non-radioactive aldosterone.

In all experiments the animals were killed by a sharp blow on the head.

#### Isolation of fractions

The isolation of renal sub-cellular fractions was by a modification of the method of Herman et al<sup>40</sup>. All isolation and fractionation procedures, unless otherwise indicated, were carried out at 0 - 4°. Kidneys were removed and homogenized in 6 volumes of 0.25 M sucrose in a teflon-glass homogenizer at 850-900 rpm, 6 strokes up and down. The renal homogenate was centrifuged for 10 min at 600 x g (2400 rpm in the 870 rotor of an International model B-20 refrigerated centrifuge). The 100,000 x g supernatant (cytosol fraction) was obtained by centrifugation of the 600 x g supernatant at 10,000 x g for 10 min and recentrifugation of the resultant 10,000 x g supernatant for 60 min at 100,000 x g (35,000 rpm in the type 50.1 rotor of a Beckman Model L Ultracentrifuge).

In in vitro experiments the 600 x g pellet (crude nuclear fraction) was suspended in 5 ml of solution "A" by gentle homogenization by hand, and stirred gently for 15 min at 0°. The nuclei were then removed by centrifugation at 20,000 x g for 15 min. The resulting supernatant was termed the 'nuclear extract'.

In in vivo experiments the nuclei were purified before being extracted with solution "A". This was done by resuspending the crude nuclear pellet in 5 ml of 2.2 M



sucrose-3 mM  $\text{CaCl}_2$  and collecting the pellet after centrifuging the suspension for 30 minutes at  $56,000 \times g$  in the Beckman Model L Ultracentrifuge using the 50.1 rotor.

Measurement of  $^3\text{H}$ -aldosterone binding to macromolecules

The binding of  $^3\text{H}$ -aldosterone to macromolecules in nuclear extracts and cytosol fractions was determined by gel filtration on Sephadex G-50 or G-75. 1 ml of nuclear extract or cytosol was applied to a K15 x 30 Sephadex column unless otherwise indicated and 3 ml fractions were collected. Protein concentrations of fractions eluted from columns were estimated spectrophotometrically by the ratio of optical densities at 280 and 260 nm<sup>87,88</sup>. The  $^3\text{H}$ -aldosterone content of the fractions was assayed by adding 1 ml aliquots of each fraction to 15 ml of solution "B". Radioactive samples were counted for 40 min at  $4^\circ$  in a liquid scintillation spectrometer (Intertechnique Model SL30 or Nuclear Chicago Mark I), at 18-22% efficiency. Quench corrections were made by recounting the samples for 1 min after addition of 50  $\mu\text{l}$  of  $^3\text{H}$ -water containing 100,000 dpm as internal standard. Results are expressed either as aldosterone concentrations (moles/ml) or as specific activities in dpm/mg protein or fmoles/mg protein.

## RESULTS

### 1. Studies on $^3\text{H}$ -Aldosterone-Protein Complexes Formed in vivo

The initial stages of this project consisted of the preliminary characterization of the aldosterone binding system and as such were mainly repetition of some of the work published by Edelman and coworkers<sup>40,71,73</sup>.

#### a. Demonstration of in vivo binding -

Figure 1 shows that when  $^3\text{H}$ -aldosterone is injected into adrenalectomized rats at concentrations similar to physiological aldosterone concentrations of normal rats, 36 percent of the  $^3\text{H}$ -aldosterone in kidney nuclear extracts is associated with protein. This compares with 48 percent reported by Edelman's group<sup>40</sup>.

A distinct separation of free from protein-bound aldosterone is evident in the figure and dissociation of the complexes on the column is minimal since very little tailing is observed.

Figure 2 shows similarly that 11 percent of the  $^3\text{H}$ -aldosterone in kidney cytosol is associated with protein. Other workers obtained 30 percent associated with protein in cytosol<sup>40</sup>. A distinct separation of free from bound aldosterone is demonstrated.

Although these results are slightly lower than those obtained by Edelman's group, a study of these aldosterone

Fig. 1.

Sephadex G-50 separation of free and bound  $^3\text{H}$ -aldosterone of nuclear extract.

1.0 ml of "solution A" extract of rat kidney nuclei labelled in vivo was applied to a Sephadex column (bed dimensions 1.5 x 22 cm), and 2.5 ml fractions were collected. Aldosterone concentrations of eluates, ●—●, were estimated from their tritium activities after quench corrections and from the specific activity of the  $^3\text{H}$ -aldosterone. Protein concentrations, ○—○, were obtained from the ratio of O.D.'s at 280 and 260 nm. Other details are as outlined in Methods.

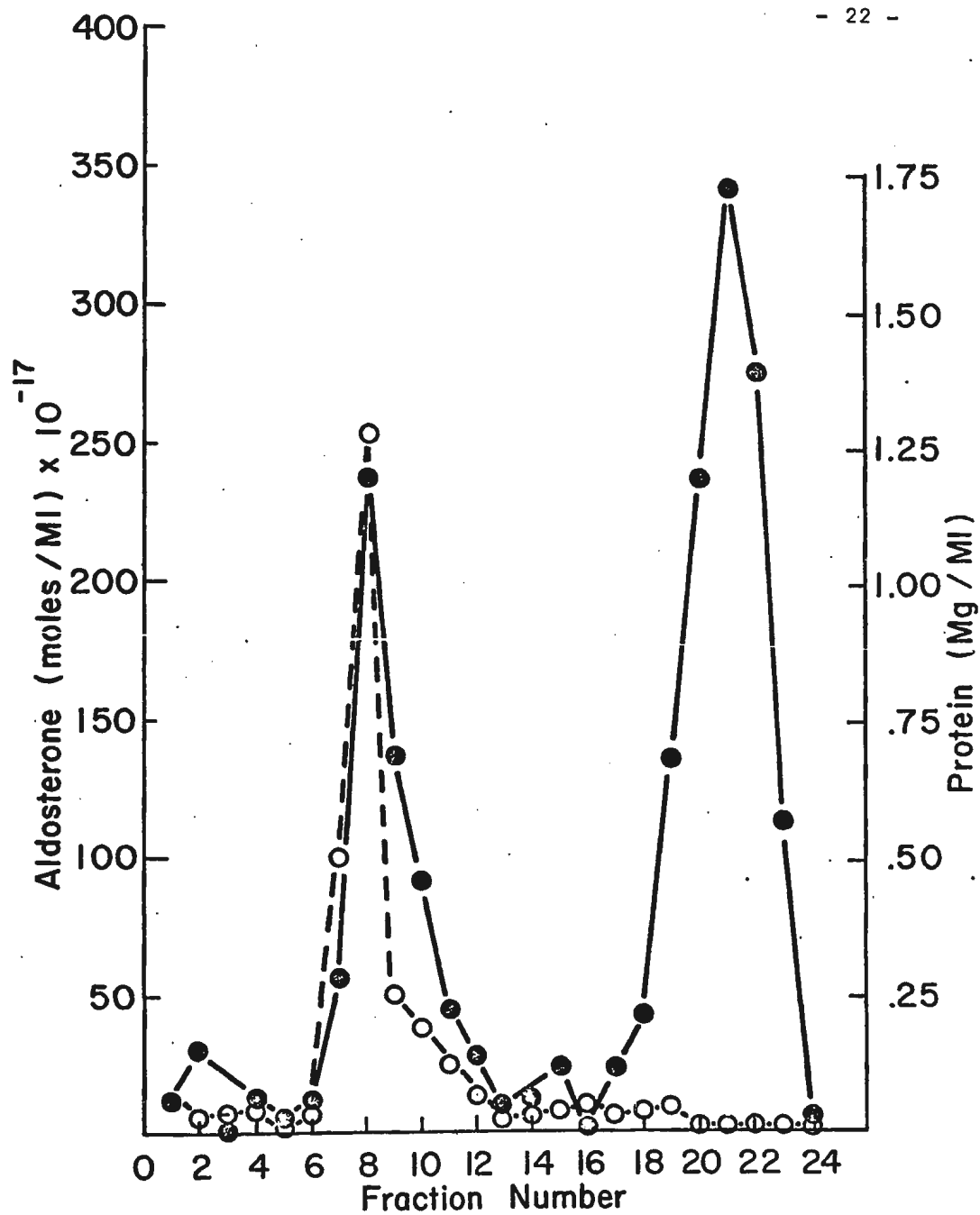


FIGURE 1

Fig. 2.

Sephadex G-50 separation of free and bound  $^3\text{H}$ -aldosterone  
of cytosol.

Conditions were as outlined for nuclear extract in Fig. 1.

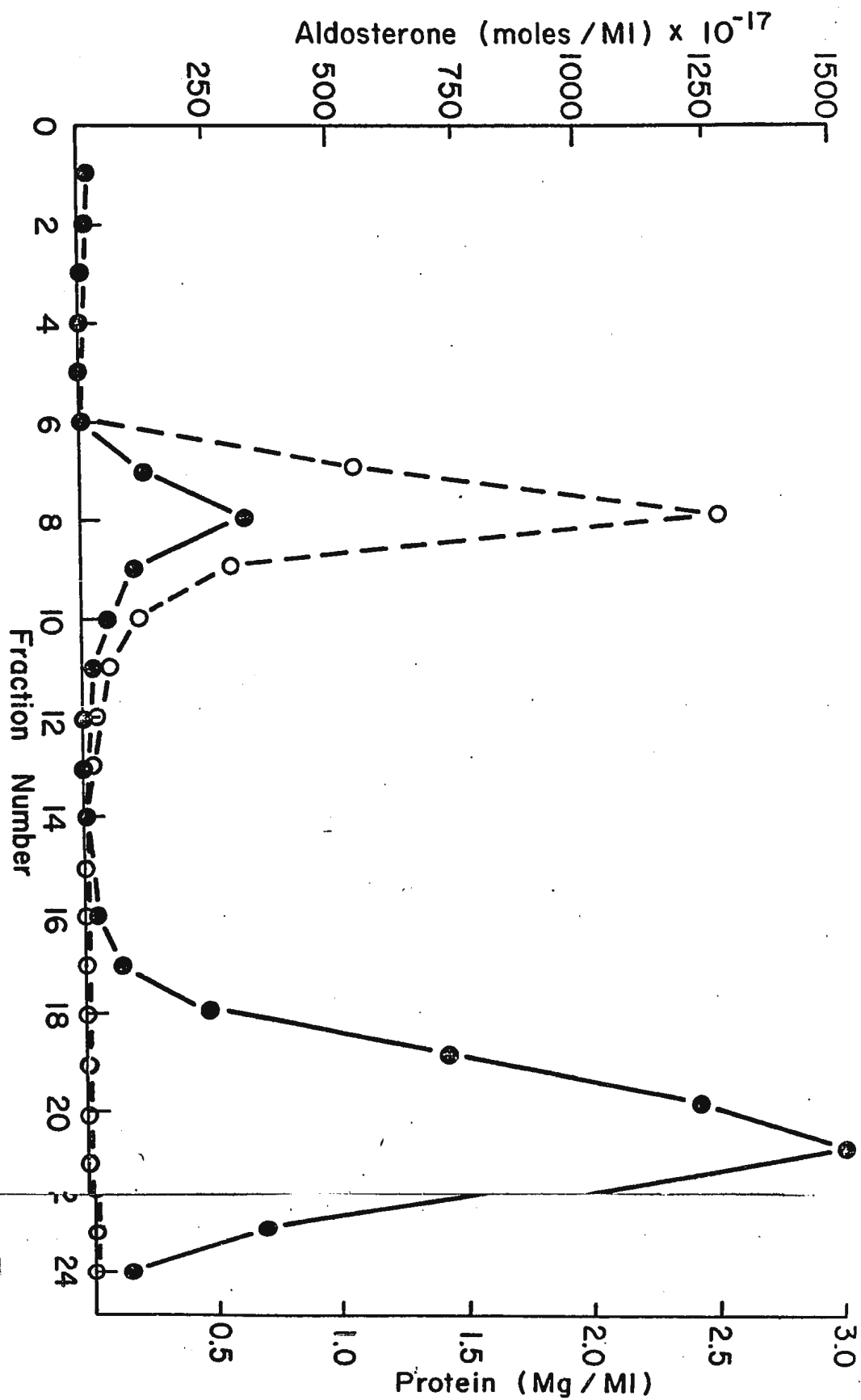


FIGURE 2

binding proteins appears feasible.

The specific activities of complexes in nuclear extract and cytosol as determined from the graphs are 2.4 and 1.4 fmoles per milligram protein respectively and hence the binding ratio of nuclear extract to cytosol is 1.7. This will be referred to elsewhere in this work.

b. Stability studies of aldosterone-protein complexes -

One problem encountered in experiments on aldosterone binding proteins is their relative instability. Aldosterone-protein complexes tend to dissociate with time and this dissociation takes place even at 0-4°.

Table I shows that the amounts of binding in some experiments were higher than the 36 percent shown in figure 1. However, the percentage of <sup>3</sup>H-aldosterone bound to protein is reduced from 48 to 28 percent when nuclear extracts remain for 5 hours at 0-4°; but the percentage is not changed significantly when the complexes are stored in the nucleus. This suggests that some nuclear factor stabilizes the binding of aldosterone to nuclear proteins. The presence of DNA or a higher intranuclear protein concentration relative to that of nuclear extracts may have a stabilizing effect on aldosterone-protein complexes.

Herman and Edelman report that <sup>3</sup>H-aldosterone-protein complexes are stabilized by 20 - 25 percent glycerol<sup>73</sup>.

TABLE I

EFFECT OF NUCLEAR ENVIRONMENT ON COMPLEX STABILITY

The homogenate of pooled kidneys (4) was obtained after injection of  $^3\text{H}$ -aldosterone and divided into 3 parts. Purified nuclear pellets were obtained and extracted with 5 ml of solution "A". See Methods for details of in vivo experiments.

- A. Nuclear extract was obtained and fractionated immediately on Sephadex G-50.
- B. Nuclear extract was obtained and stored at  $0-4^\circ\text{C}$  for 5 hr before fractionation.
- C. The nuclear pellet was stored at  $0-4^\circ\text{C}$  for 5 hr before extraction. The extract was fractionated immediately.

	A	B	C
Percentage bound $^3\text{H}$ -aldosterone	48	28	50



Figure 3 shows that in our hands the increased stability afforded by 20 percent glycerol to complexes of nuclear extracts was slight. The effect was detectable at 20 and 25 hours after nuclear extract isolation. However, it was felt that routine use of this technique was not worthwhile.

c. Temperature and pH dependence -

In confirmation of the results published by Edelman's group<sup>73</sup>, we demonstrated that complexes were sensitive to changes in pH and temperature. In nuclear extracts isolated after <sup>3</sup>H-aldosterone injection, we found that lowering the pH to 6.3 reduced complex activity to 30 percent that of controls at pH 7.4. Elevating the pH to 8.4 reduced activity to 60 percent. Incubation of nuclear extract for 20 minutes at 35° reduced activity to 75 percent that of the control.

d. Effect of hyaluronidase -

Incubation of nuclear extract with hyaluronidase (800 µg/ml final concentration) for 15 minutes at 0-4° reduced the specific activity of complexes by less than 10 percent. This suggests that either the aldosterone binding protein is not a hyaluronic acid containing glycoprotein or that the splitting of hyaluronic acid from the protein does not affect the binding of <sup>3</sup>H-aldosterone significantly. Fanestil and Edelman found that neuraminidase inhibited the binding of <sup>3</sup>H-aldosterone to proteins in nuclear extracts by

Fig. 3.

Stabilization of  $^3\text{H}$ -aldosterone-protein complexes by glycerol.

Nuclear extract from the pooled kidneys of 2 rats injected with  $^3\text{H}$ -aldosterone was divided into two aliquots. Glycerol to 20% by volume was added to the test, o—o, and a similar addition of diluent (Solution A) was made to the control, ●—●. The test was eluted with Solution A made to 20% glycerol by volume and the control was eluted with Solution A. 100% is activity of the control at 0 time, 290dpm/mg.

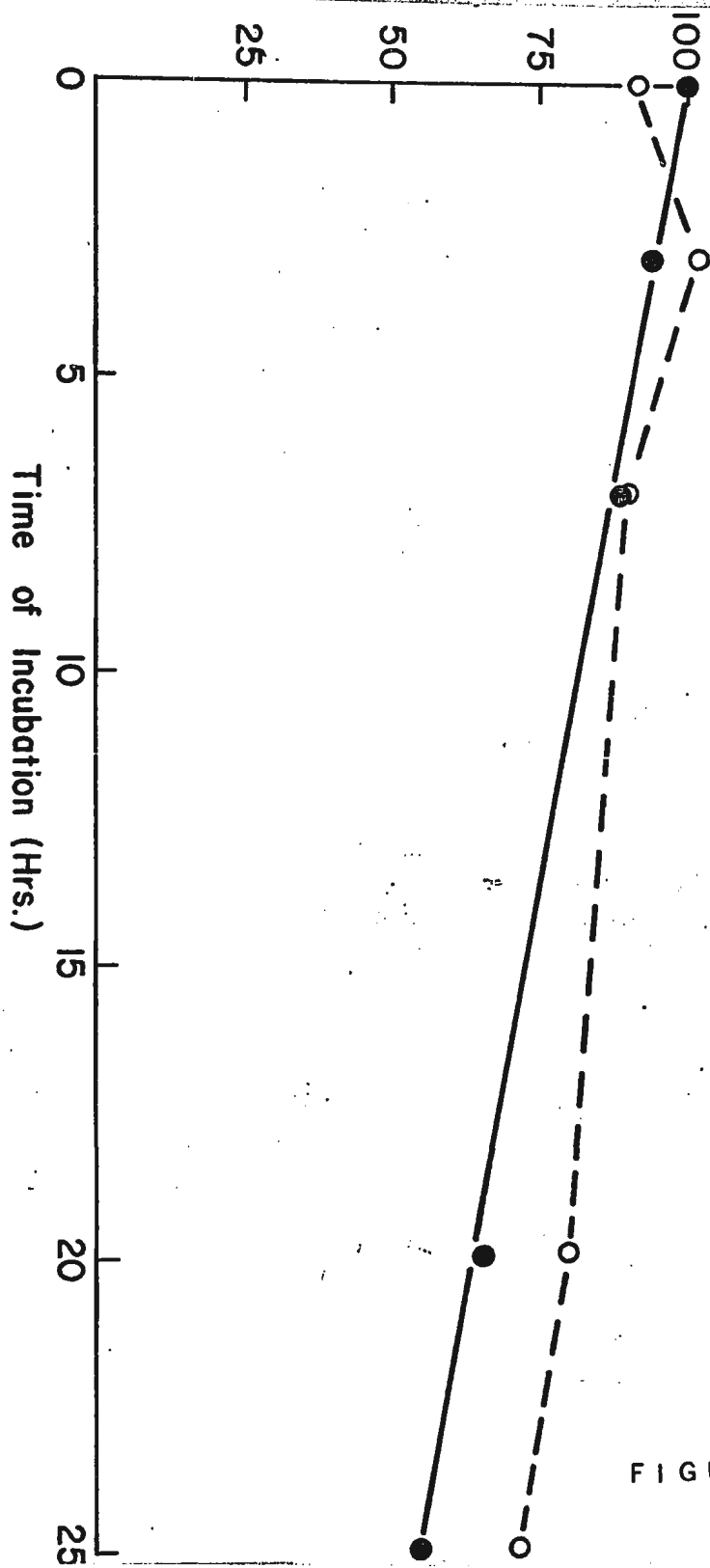


FIGURE 3

6 percent<sup>71</sup>. Hence it appears that the aldosterone binding protein of rat kidney nuclear fraction does not possess a carbohydrate moiety that is functional in the binding of aldosterone.

## 2. Studies of <sup>3</sup>H-aldosterone-Protein Complex Formation in vitro

Having confirmed that we could obtain results similar to those of Edelman and co-workers, we continued with the project. Attempts were made to characterize aldosterone binding proteins by studying the formation of complexes in vitro and in addition by comparing in vivo and in vitro binding.

### a. Inhibition by p-HMB -

P-HMB has been shown to destroy <sup>3</sup>H-aldosterone-protein complexes formed in vivo<sup>40</sup>. Figure 4 shows that p-HMB inhibits the formation of <sup>3</sup>H-aldosterone-protein complexes in vitro. The inhibition increases sharply with p-HMB concentration up to approximately 0.25 mM but at 0.50 mM little further increase in inhibition is observed.

The residual binding of <sup>3</sup>H-aldosterone shown in Figure 4 may result either from two proteins binding aldosterone of which one is little affected by p-HMB or alternately, there could be one protein whose affinity for aldosterone is considerably reduced by p-HMB. The latter suggestion is less likely since the expected tailing due to dissociation on the column is not observed.

Fig. 4.

Effect of p-HMB on  $^3\text{H}$ -aldosterone uptake by protein in isolated renal nuclear extract.

Initial incubations with the indicated concentrations of p-HMB were for 10 min at  $0-4^{\circ}$ .  $^3\text{H}$ -aldosterone was then added and the incubations continued for 2.5 hr. 100% is the activity in the absence of p-HMB (245 dpm/mg protein).

- 29 -  
Specific Activity (% of Control)

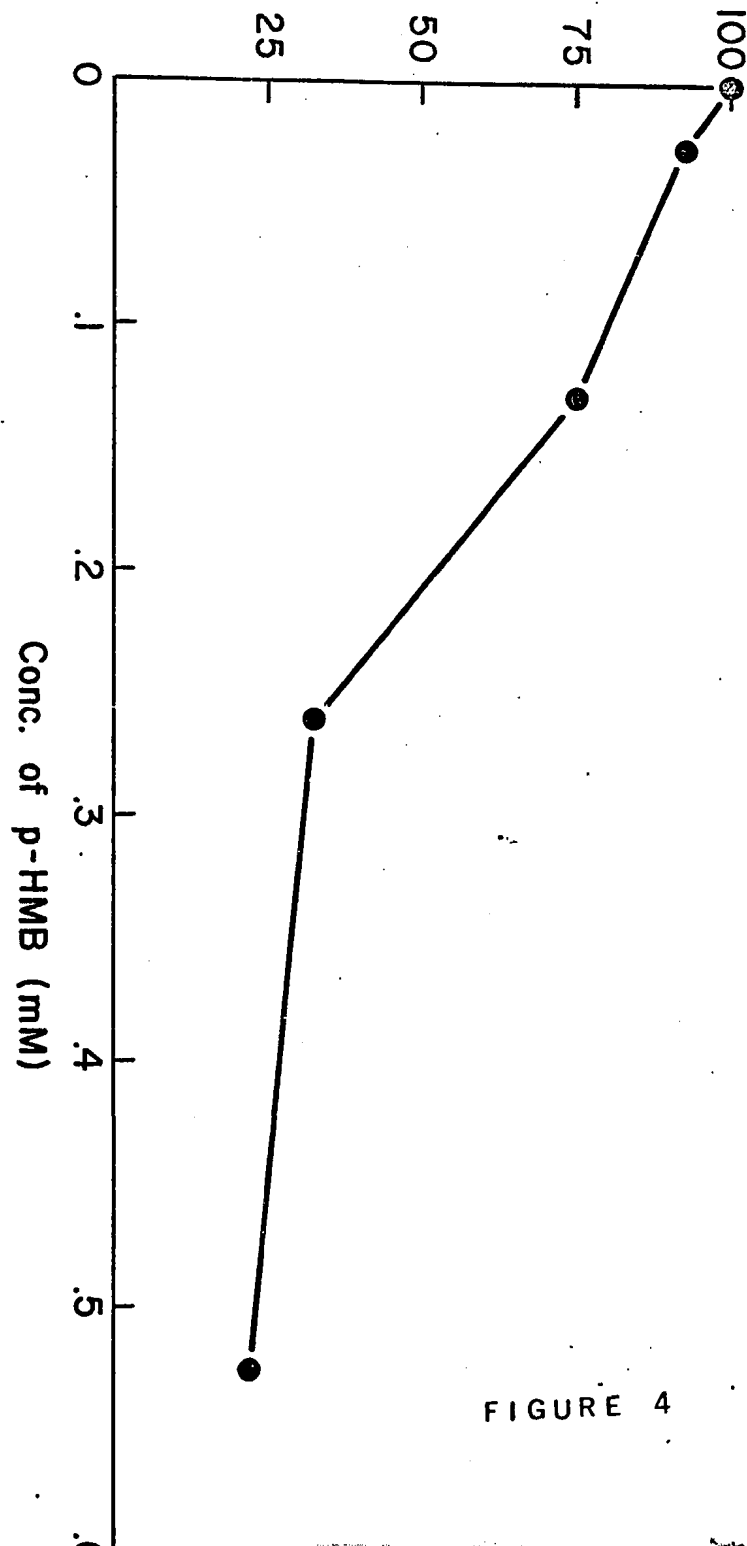


FIGURE 4

b. Aldosterone protection against p-HMB inhibition -

The possibility that aldosterone might afford some protection against p-HMB inhibition was investigated. The results are shown in Table II. Pre-incubation of nuclear extract with  $^3\text{H}$ -aldosterone does not protect the complexes from dissociation by p-HMB. Apparently in the presence of p-HMB the amount of binding by aldosterone to protein is markedly reduced.

c. Ratio of nuclear to cytosol binding in vivo and in vitro -

Table III indicates that specific activities of complexes formed in vitro are greater than those of complexes formed in vivo in both nuclear extracts and cytosol fractions. However, the in vivo nuclear extract to cytosol binding ratio (1.9) is greater than the in vitro ratio (0.54). This suggests that either aldosterone-protein complexes move to the nucleus after injection of aldosterone or alternately that the nuclear and cytosol binding proteins decay at different rates after isolation.

d. Effect of bovine serum albumin on aldosterone binding -

As mentioned earlier in 1.b where it was shown that complexes are more stable when stored in the nucleus, the existing intranuclear protein concentration may stabilize nuclear binding of aldosterone. If such were the case, it might explain the higher in vivo nuclear extract to cytosol

TABLE II

## ALDOSTERONE PROTECTION AGAINST p-HMB INHIBITION

The final concentration of  $^3\text{H}$ -aldosterone was  $5 \times 10^{-10}$  M (50,000 dpm) in each case and p-HMB concentration was 0.132 mM. Sephadex G-50 chromatography was carried out 2 hr after the final addition of  $^3\text{H}$ -aldosterone. All incubations were at  $0^\circ$ .

<u>Initial additions</u>	<u>Additions after 2 hr</u>	<u>Specific activity of complex (dpm/mg protein)</u>	<u>% activity (of control)</u>
N.E. (0.958 ml) + $^3\text{H}$ -aldosterone (0.042 ml)	-	345	100
N.E. (0.933 ml) + $^3\text{H}$ -aldosterone (0.042 ml)	p-HMB (0.025 ml)	270	78
N.E. (0.933 ml)	p-HMB (0.025 ml) + $^3\text{H}$ -aldosterone (0.042 ml)	265	77
N.E. (0.933 ml) + $^3\text{H}$ -aldosterone (0.021 ml)	p-HMB (0.025 ml) + $^3\text{H}$ -aldosterone (0.021 ml)	260	76
N.E. (0.933 ml) + $^3\text{H}$ -aldosterone (0.010 ml)	p-HMB (0.025 ml) + $^3\text{H}$ -aldosterone (0.032 ml)	260	76



TABLE III

<sup>3</sup>H-ALDOSTERONE-PROTEIN COMPLEXES IN RAT KIDNEY NUCLEAR  
EXTRACTS AND CYTOSOL.

The number of experiments for which mean values were taken is indicated in parentheses. The kidneys from two rats were pooled in each experiment. See Methods for details of experiments.

	Specific Activity (dpm/mg protein)		N.E. Cytosol
	N.E.	Cytosol	
<u>In vivo</u> (2)	183	96	1.90
<u>In vitro</u> (5) ( <sup>3</sup> H-aldosterone added after isolation)	253	468	0.54

binding ratio obtained in 2.c. The possibility that higher protein concentrations may protect aldosterone binding was investigated by adding bovine serum albumin to isolated fractions immediately prior to adding  $^3\text{H}$ -aldosterone.

The data presented in Table IV has been corrected for binding to B.S.A. and indicates that addition of B.S.A. to nuclear extracts increases the binding of  $^3\text{H}$ -aldosterone by 60 percent. Cytosol binding was little affected by B.S.A. The resulting increase in the nuclear extract to cytosol binding ratio indicates that binding in nuclear extracts may be partially dependent on protein concentration.

e. Competition between endogenous and exogenous aldosterone  
in vivo and in vitro -

Table V offers evidence that at least some endogenous aldosterone is bound to macromolecules. In in vivo experiments employing adrenalectomized rats, specific activities of  $^3\text{H}$ -aldosterone-protein complexes in cytosol fractions and nuclear extracts were 3- and 4-fold greater respectively than in corresponding experiments using control animals. Experiments performed in vitro show specific activities (dpm/mg protein) of 405 for nuclear extracts of kidneys from adrenalectomized animals and 224 for extracts from control animals. Cytosol activities were 939 for adrenalectomized animals and 320 for controls. The specific activities of complexes in both fractions appear increased approximately 2-fold in adrenalectomized rats.

TABLE IV

EFFECT OF BOVINE SERUM ALBUMIN (B.S.A.) ON  $^3\text{H}$ -ALDOSTERONE  
BINDING TO NUCLEAR EXTRACT AND CYTOSOL.

B.S.A. (final concentration 4.5 mg/ml) was added to isolated nuclear extract and cytosol immediately before addition of  $^3\text{H}$ -aldosterone [+]. Only  $^3\text{H}$ -aldosterone was added to the controls [-]. See Methods for details of in vitro experiments.

	<u>Specific Activity</u> <u>dpm/mg protein</u>		
	<u>-</u>	<u>+</u>	<u>Ratio</u>
N.E.	209	337	1.62
Cytosol	310	303	0.98
B.S.A. (alone)	22		
<u>N.E.</u>	0.67	1.11	
<u>Cytosol</u>			

TABLE V

ALDOSTERONE BINDING IN ADRENALECTOMIZED AND UNOPERATED ANIMALS

Pooled kidneys from two adrenalectomized rats and from two unoperated controls were employed in the in vivo experiments. In vitro values are averages of two experiments, each employing two adrenalectomized rats and two unoperated controls. See Methods for experimental details.

	<u>Specific activity (dpm/mg protein)</u>	
	<u>N.E.</u>	<u>Cytosol</u>
<u>In vivo</u> -		
Adrenalectomized rats	210	120
Control rats	50	40
<u>In vitro</u>		
( <sup>3</sup> H-aldosterone in isolation medium)		
Adrenalectomized rats	405	939
Control rats	224	320

These results suggest that in normal intact animals some steroids produced by the adrenal cortex are bound to protein. The amount of  $^3\text{H}$ -aldosterone which may bind to protein in these intact animals is therefore reduced by competition. Inhibition by exogenous corticosteroids will be demonstrated in section 3.c.

f. In vitro binding after adrenalectomy -

The previous section demonstrated that compared to activities in kidney fractions of control animals, the specific activities of complexes are increased in kidney fractions from animals that have been adrenalectomized 6-10 days before sacrifice. Now we asked the question "At what time after adrenalectomy do these increases first appear?" The results of a time course of adrenalectomy study are presented in Figures 5 and 6.

Figure 5 shows that at one hour after adrenalectomy there is a slight increase in complex activity of nuclear extracts. At two hours after surgery, however, a 3-fold increase in specific activity is observed. Although considerable scatter is apparent, the tendency for a 2-3 fold increase in activity remains, up to 28 days after the operation.

Figure 6 shows that at one hour after surgery the specific activity of cytosol complexes is twice that of the control. Only a slight further increase in activity is noted up to 28 days after adrenalectomy. A considerable amount of

Fig. 5.

Effect of adrenalectomy on binding in nuclear extract.

Rats were adrenalectomized and sacrificed at intervals ranging from 1 hr to 28 days after surgery.  $^3\text{H}$ -aldosterone was included in the isolation and nuclear extraction media. Other experimental details are as outlined in Methods.

Each experiment employed the pooled kidneys of two rats and each point on the graph represents the mean of two experiments. A value for unoperated control rats, which is the average of six experiments, is represented by the point, X.

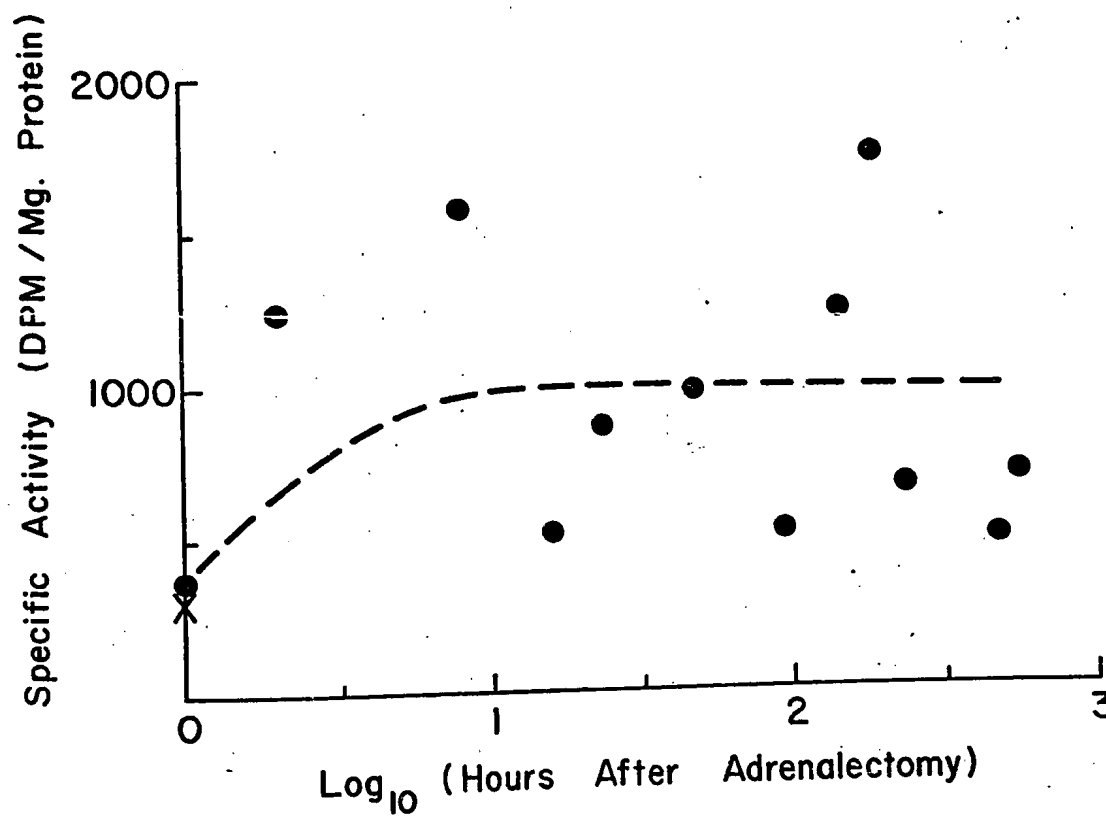


FIGURE 5

Fig. 6.

Effect of adrenalectomy on binding in cytosol.

Conditions were as outlined for nuclear extract in  
Fig. 5.



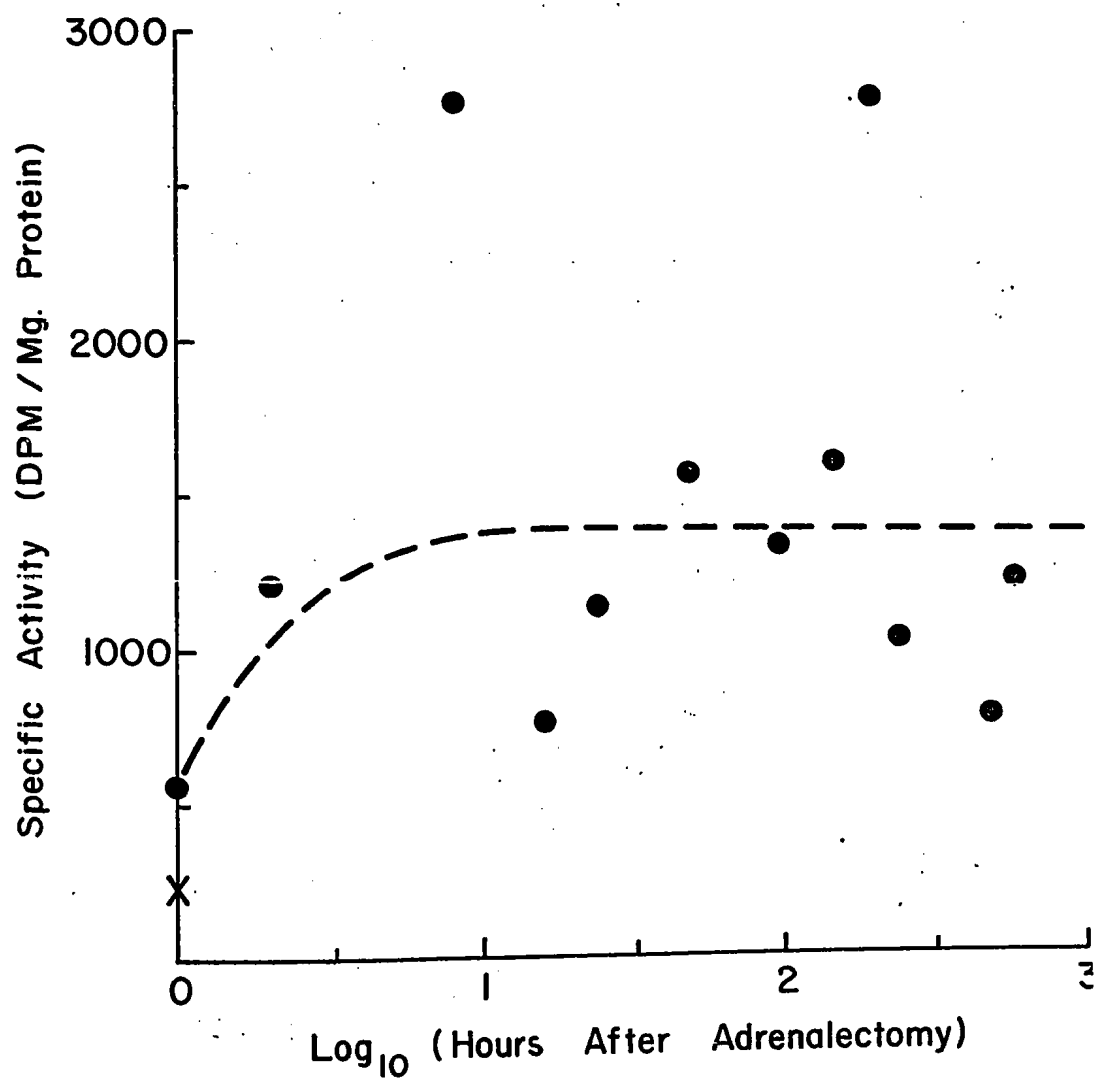


FIGURE 6

scatter is observed again. This is probably due to variations in individual animals.

### 3. Evidence for Heterogeneity of Aldosterone Binding Proteins

As discussed in the Introduction, the properties of mineralocorticoid-binding proteins isolated from the kidney and estrogen-binding proteins of the uterus are impressively similar and evidence of heterogeneity of estrogen-binding proteins has been discussed by Jensen et al<sup>89</sup>. Heterogeneity of the aldosterone binding system has been suggested from the kinetics of uptake of <sup>3</sup>H-aldosterone by rat kidney nuclei in vivo and from the binding of <sup>3</sup>H-aldosterone to the intact urinary bladder of the toad<sup>70,71</sup>. The apparent heterogeneity may however be due to cooperative interactions rather than to multiple binding proteins. Some of the subsequent work described here seems to offer additional evidence in support of multiple binding proteins for aldosterone.

#### a. Demonstration of increased complex activity by inclusion of <sup>3</sup>H-aldosterone in the isolation medium -

Matthysse and Phillips<sup>90</sup> have shown that a protein factor may be lost from tobacco and soybean nuclei unless they are isolated in the presence of the plant hormone auxin. Are aldosterone binding proteins lost from kidney nuclei when these organelles are isolated in vitro in the absence of aldosterone? If such were the case, the lower nuclear extract to cytosol

binding ratio demonstrated in 2.c would be predicted because in the absence of hormone, the binding proteins lost from the nucleus may appear as cytosol binding proteins or alternatively may lose entirely their ability to bind aldosterone.

The hypothesis that aldosterone binding proteins were somewhat analogous to the auxin protein factors was tested and the results are presented in Table VI. Inclusion of  $^3\text{H}$ -aldosterone in the isolation and nuclear extraction media of rat kidney increases the binding of  $^3\text{H}$ -aldosterone to proteins in both nuclear extracts and cytosol fractions. The marked stimulation at zero time may be partially attributed to the longer incubation time of  $^3\text{H}$ -aldosterone with fractions when hormone is present in the isolation medium. However, after 2.5 hr incubation, the 1.7-fold stimulation of nuclear extract binding and the 3-fold stimulation of cytosol binding suggest definite increases in activity since maximum in vitro binding has been reported to occur at 2-2.5 hr<sup>40</sup>.

b. Inhibition of in vitro binding by  $17\beta$ -estradiol—

The stimulation of binding by inclusion of aldosterone in the isolation medium raises the question of whether or not the increased binding under these conditions and the binding in pre-isolated fractions involve identical binding moieties. It has been shown by other workers<sup>40</sup> and we confirm in Table VII that in vitro aldosterone binding to pre-isolated nuclear extracts and cytosol fractions is not inhibited by

TABLE VI

EFFECT OF ADDING  $^3\text{H}$ -ALDOSTERONE TO ISOLATION MEDIUM

0 time was defined as the time of isolation of fractions.

$^3\text{H}$ -aldosterone was added immediately after isolation to controls [-], and to the homogenization medium and Solution "A" extraction medium in the tests [+]. Each experiment employed one rat as control and one as test and the figures shown are the mean of three experiments. Other conditions of the experiment were as outlined in Methods.

	Specific Activity dpm/mg protein		Ratio (+/-)
	-	+	
N.E. (0 hr)	91	413	4.5
Cytosol (0 hr)	99	1230	12.4
N.E. (2.5 hr)	258	437	1.7
Cytosol (2.5 hr)	680	2040	3.0

TABLE VII

INHIBITION BY ESTRADIOL-17 $\beta$

<sup>3</sup>H-aldosterone (final concentration  $5 \times 10^{-10}$  M) was added to the controls [-] and <sup>3</sup>H-aldosterone (final concentration  $5 \times 10^{-10}$  M) plus 17 $\beta$ -estradiol (final concentration  $10^{-8}$  M) was added to tests [+]. Values shown are means of two experiments each employing pooled kidneys from two animals. See Methods for outline of experimental conditions.

	Specific Activity dpm/mg protein		Percent Inhibition
	-	+	
Hormones added to isolation media -			
N.E.	1015	780	23
Cytosol	1580	907	43
Hormones added after isolation of fractions -			
N.E.	480	450	6
Cytosol	648	615	5

17 $\beta$ -estradiol. However, Table VII also shows that binding is inhibited 23 percent in nuclear extracts and 43 percent in cytosol when 17 $\beta$ -estradiol is included with <sup>3</sup>H-aldosterone in the isolation media. These results suggest that in addition to the aldosterone specific binding protein, rat kidney also contains an aldosterone binding protein which binds 17 $\beta$ -estradiol.

c. Inhibition of in vitro binding by other steroids -

Since binding seems to be increased when <sup>3</sup>H-aldosterone is included in the isolation medium and this increased binding is inhibited by 17  $\beta$ -estradiol, a more detailed study of inhibition under the two conditions of binding was undertaken.

Tables VIII and IX present the results of such a study. Table VIII shows that inhibition of aldosterone binding takes place with all steroids tested. Deoxycorticosterone is the most potent inhibitor of complex formation in both nuclear extract and cytosol. The other steroids used in the experiment inhibit complex formation by 20 to 40 percent. It should be noted that controls employing "crossed kidneys" (as explained in Table VIII) were run in all experiments since the amount of binding in kidney fractions varies significantly for individual rats.

Table IX shows that testosterone and cortisol inhibit binding in nuclear extracts by less than 10 percent. Again

TABLE VIII

## INHIBITION OF ALDOSTERONE BINDING BY HORMONAL STEROIDS - I

Kidneys from two rats were used in each experiment. A kidney from rat A was combined with a kidney from rat B and the combination was used as the control.\* The remaining two kidneys were used as the test.  $^3\text{H}$ -aldosterone (final concentration  $5 \times 10^{-10}$  M) was added to the isolation media of controls.  $^3\text{H}$ -aldosterone (final concentration  $5 \times 10^{-10}$  M) plus an additional steroid (final concentration  $5 \times 10^{-8}$  M) were added to the isolation media of tests. After isolation, the fractions were incubated for 2.5 hr at  $0-4^\circ$  before assay.

Experiment No.	Steroids added	Specific Activity (dpm/mg protein)		Percent Inhibition	
		N.E.	Cytosol	N.E.	Cytosol
1	$^3\text{H}$ -aldosterone	1062	2260		
	$^3\text{H}$ -aldosterone + progesterone	734	1570	31	30
2	$^3\text{H}$ -aldosterone	2220	2820		
	$^3\text{H}$ -aldosterone + testosterone	1450	2260	35	20
3	$^3\text{H}$ -aldosterone	1840	3890		
	$^3\text{H}$ -aldosterone + cortisone	1110	3440	40	13
4	$^3\text{H}$ -aldosterone	241	795		
	$^3\text{H}$ -aldosterone + cortisol	193	613	20	23
5	$^3\text{H}$ -aldosterone	1525	3115		
	$^3\text{H}$ -aldosterone + DOC	540	880	65	72

\* This method of "crossing" kidneys gave results that were reproducible within 5%.

TABLE IX

## INHIBITION OF ALDOSTERONE BINDING BY HORMONAL STEROIDS - II

Pooled kidneys from two rats were used in each experiment.  $^3\text{H}$ -aldosterone alone (final concentration  $5 \times 10^{-10}$  M) or  $^3\text{H}$ -aldosterone plus the indicated steroid (final concentration  $5 \times 10^{-8}$  M) were added to isolated nuclear extracts and cytosol fractions and incubations at  $0-4^\circ$  for 2.5 hr were carried out before assay.

Experiment No.	Steroids added	Specific Activity (dpm/mg protein)		Percent Inhibition	
		N.E.	Cytosol	N.E.	Cytosol
1	$^3\text{H}$ -aldosterone	125	480		
	$^3\text{H}$ -aldosterone + progesterone	100	390	20	19
2	$^3\text{H}$ -aldosterone	110	650		
	$^3\text{H}$ -aldosterone + testosterone	100	410	9	37
3	$^3\text{H}$ -aldosterone	100	465		
	$^3\text{H}$ -aldosterone + cortisone	55	370	45	20
4	$^3\text{H}$ -aldosterone	578	820		
	$^3\text{H}$ -aldosterone + cortisol	574	516	< 1	37
5	$^3\text{H}$ -aldosterone	165	585		
	$^3\text{H}$ -aldosterone + DOC	80	190	51	68



DOC is the most potent inhibitor in both fractions. Individual variations can again be seen and controls were run in each experiment.

Tables VIII and IX suggest that under both conditions of isolation (i.e. in the presence or in the absence of aldosterone), binding is inhibited to the greatest extent by mineralocorticoids. It is seen that in both cases, proteins of the cytosol fraction bind  $^3\text{H}$ -aldosterone with a higher specific activity than those of the nuclear extract; and in isolated fractions, inhibition is generally greater in cytosol fractions than in nuclear extracts. Finally, inhibition by hormonal steroids is less in pre-isolated fractions than in cases where hormones are added to the isolation medium.

On the basis of these studies it seems reasonable to conclude that rat kidney contains at least two aldosterone binding proteins, only one of which is relatively specific for mineralocorticoids.

d. Decay of aldosterone-binding proteins in cytosol -

Figs. 7 and 8 show that aldosterone offers some degree of protection against complex dissociation with time. When either homogenates or isolated fractions are stored, aldosterone must be present at the early stages of the isolation procedure for maximum effect.

Fig. 7. Decay of aldosterone-binding components (isolated fractions stored).

Six male rats were employed in this experiment. The kidneys from each rat were cut into thirds and one-third kidney from each rat was combined with one-third kidney from each of the other five rats. This process was continued for all kidney pieces so that six mixtures of kidney were obtained. Three were used for conditions described in Fig. 8 and the remaining three were used under the conditions described below. Zero time was the time of homogenization and the fractions were assayed at the indicated times. 100 percent was the activity at 4 hrs.

- Kidney was homogenized in the presence of  $^3\text{H}$ -aldosterone, cytosol was prepared immediately, and aliquots were assayed at the indicated times.
- ▲—▲ Kidney was homogenized and cytosol was prepared immediately.  $^3\text{H}$ -aldosterone was added to aliquots of cytosol 2 1/2 hrs before the indicated assay times.
- X—X Kidney was homogenized and cytosol was prepared immediately.  $^3\text{H}$ -aldosterone was added to the cytosol and aliquots were assayed at the indicated times.

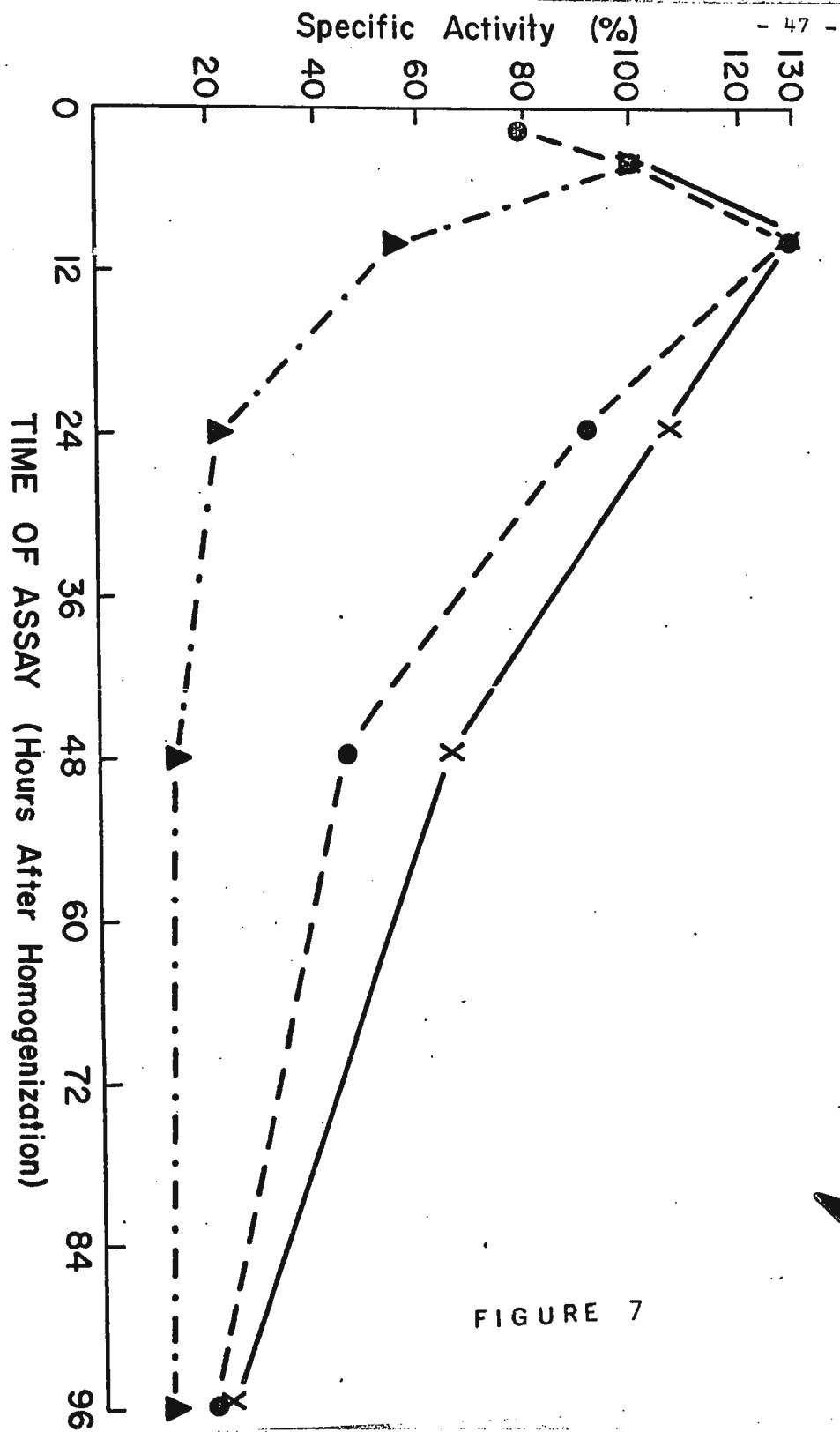


FIGURE 7

Fig. 8. Decay of aldosterone-binding components  
(homogenate stored).

Partial description of this experiment is given in Fig. 7.

- Kidney was homogenized in the presence of  $^3\text{H}$ -aldosterone and stored at  $0^\circ$ . At intervals, aliquots of homogenate were taken for preparation of cytosol.
- ~~X—X~~ Kidney homogenate was stored at  $0^\circ$ .  $^3\text{H}$ -aldosterone was added 2 1/2 hr before preparation of cytosol, i.e. 4 hr before assay.
- ▲—▲ Kidney homogenate was stored at  $0^\circ$ . Aliquots were taken at intervals for preparation of cytosol.  $^3\text{H}$ -aldosterone was added to cytosol 2 1/2 hr before assay.

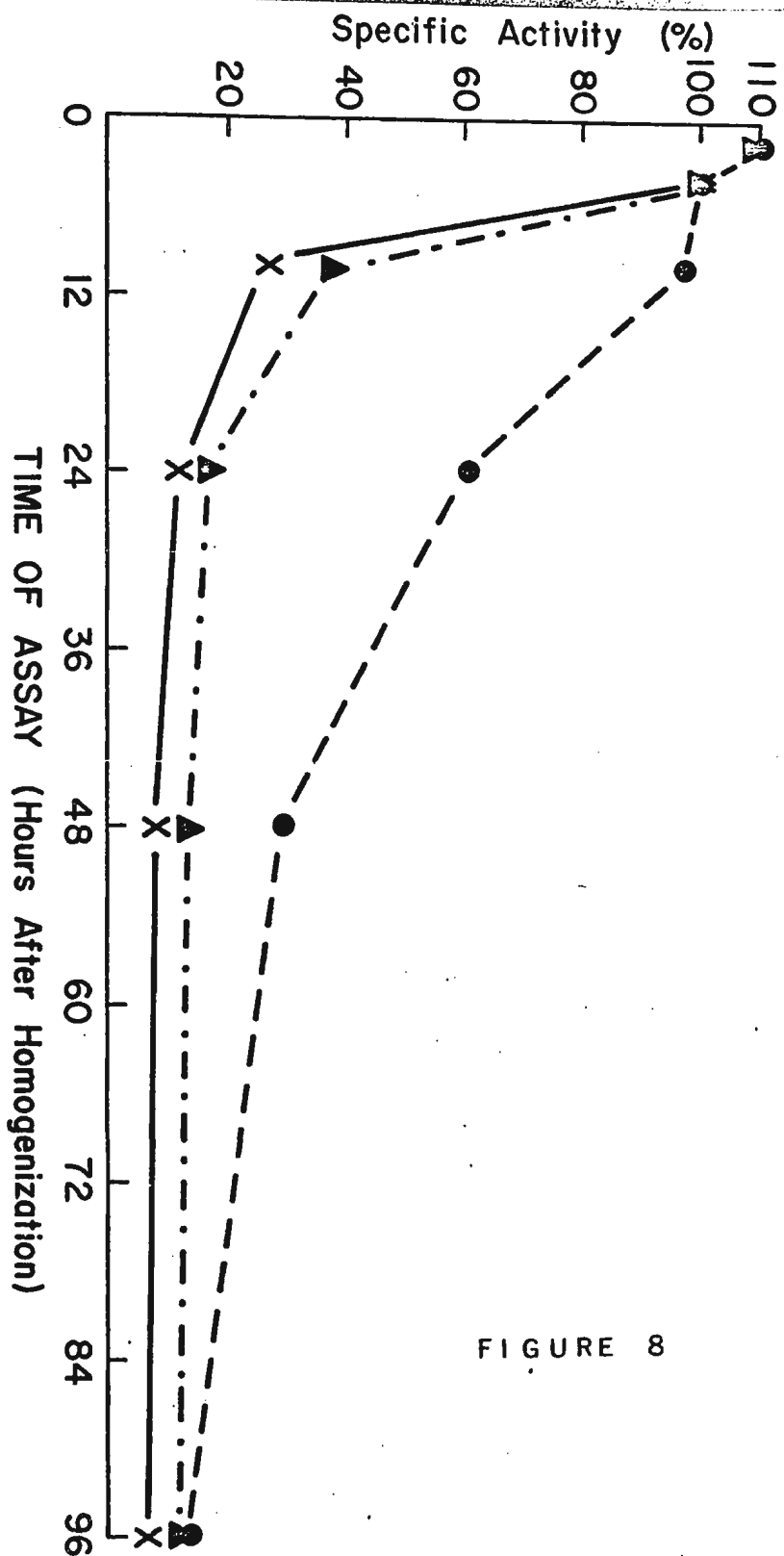


FIGURE 8

#### 4. Kinetics of Aldosterone Binding in vitro

In all the previous experiments described here and in those described by Edelman<sup>40</sup>, physiological concentrations of aldosterone were employed ( $5$  to  $6 \times 10^{-10}$  M). This is well below the plasma aldosterone concentration required to produce maximum sodium retention in vivo,  $5 \times 10^{-9}$  M. Because of this and because our results suggest heterogeneity of aldosterone binding proteins, we investigated the effects of adding higher concentrations of  $^3\text{H}$ -aldosterone on the binding of aldosterone in vitro. Double reciprocal plots under the two conditions of in vitro binding are presented here.

##### a. Double reciprocal plots for nuclear extract -

##### (i) $^3\text{H}$ -aldosterone added after isolation -

For binding in pre-isolated nuclear extracts, concentrations of aldosterone ranging from  $10^{-8}$  M to  $3 \times 10^{-7}$  M were considered. A double reciprocal plot for binding at these concentrations of aldosterone is presented in Fig. 9. A straight line plot was obtained indicating that the binding moieties for aldosterone may be homogeneous or that they have similar affinities for aldosterone.

The similarity of these double reciprocal plots to Lineweaver-Burk plots for enzymes has been outlined in the Appendix. Hence by obtaining the maximum binding from the graph, we may deduce the dissociation constant for aldosterone-macromolecular complexes. The dissociation constant for complexes in nuclear extracts appears to be  $10^{-6}$  M.

Fig. 9.

Double reciprocal plot for nuclear extract (aldosterone added after isolation).

The nuclear pellet from the pooled kidneys of 4 rats was extracted with 15 ml of Solution A.  $^3\text{H}$ -aldosterone was added to aliquots of the isolated nuclear extract to give final concentrations ranging from  $10^{-8}$  to  $3 \times 10^{-7}$  M. Other experimental details are as outlined in Methods.

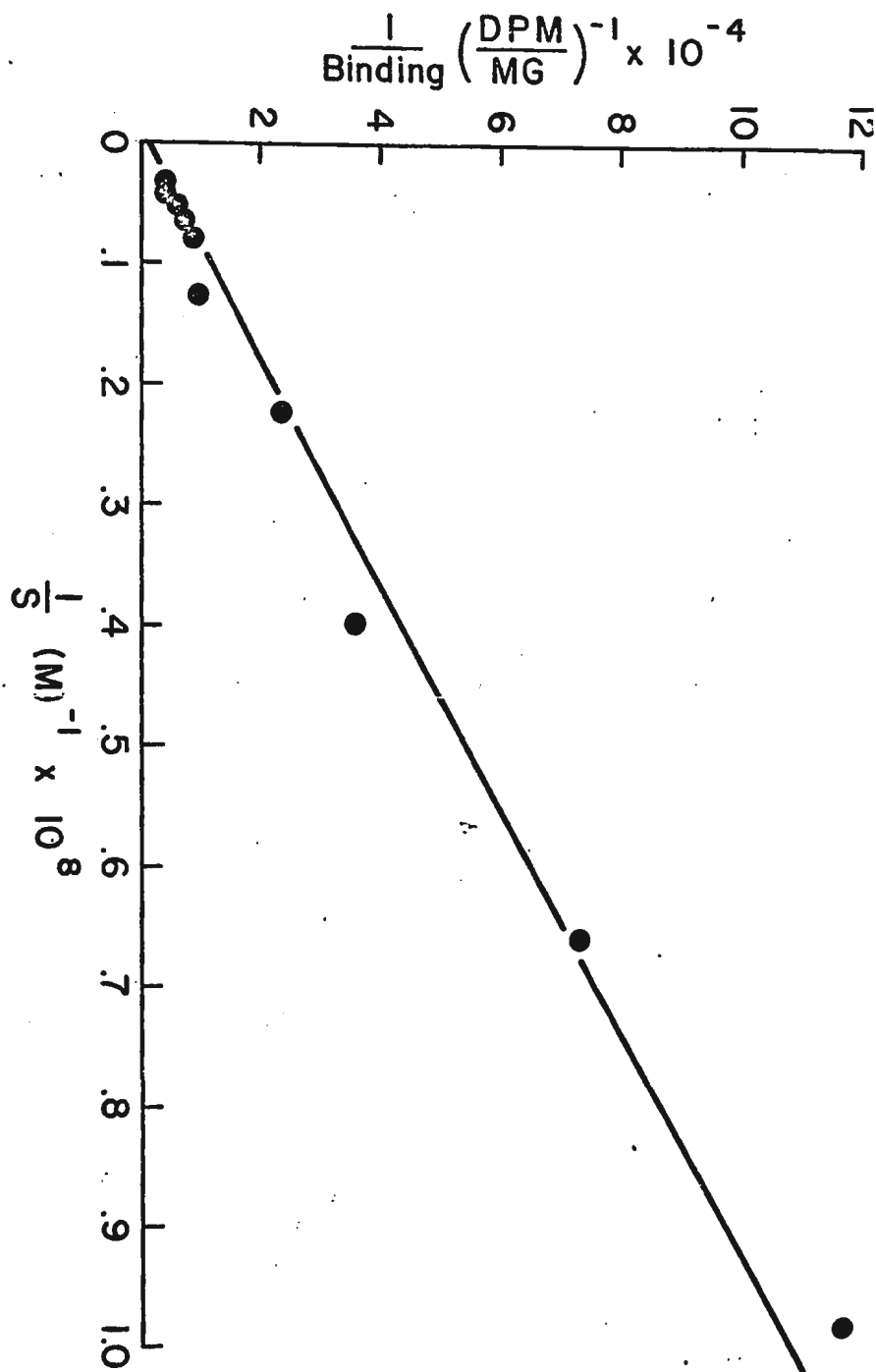


FIGURE 9



- (ii) <sup>3</sup>H-aldosterone added to isolation and nuclear extraction media -

Fig. 10 shows that the nuclear extract binding moieties do not yield a straight line double reciprocal plot when aldosterone is included in the isolation medium. This suggests that the aldosterone binding moieties are heterogeneous. Alternately, the binding proteins may be homogeneous but have interacting binding sites for aldosterone.

b. Double reciprocal plots for cytosol -

Figs. 11 and 12 show double reciprocal plots for cytosol which are similar to those shown in Figs. 9 and 10 for nuclear extract. This indicates that cytosol gives results which are analogous with those of nuclear extracts. The dissociation constant for cytosol complexes was  $2 \times 10^{-7}M$  and heterogeneity of binding proteins is again suggested when aldosterone is included in the isolation media.

These results suggest that rat kidney contains at least two macromolecular moieties which bind aldosterone. The data suggests, in addition, that the binding moieties in nuclear extract and cytosol may be different since the dissociation constants of complexes in the respective pre-isolated fractions differ by a factor of 5.

Fig. 10.

Double reciprocal plot for nuclear extract (aldosterone added to the isolation media).

Six rats were employed in this experiment. The kidneys from each rat were cut into thirds and one-third kidney from each rat was combined with one-third kidney from each of the other 5 rats. This process was continued for all kidney pieces so that binding variations in individual animals were minimized. <sup>3</sup>H-aldosterone was added to the isolation media of the 6 lots of pooled kidney to give final concentrations ranging from  $10^{-8}$  to  $10^{-7}$  M. Other experimental details are as outlined in Methods.

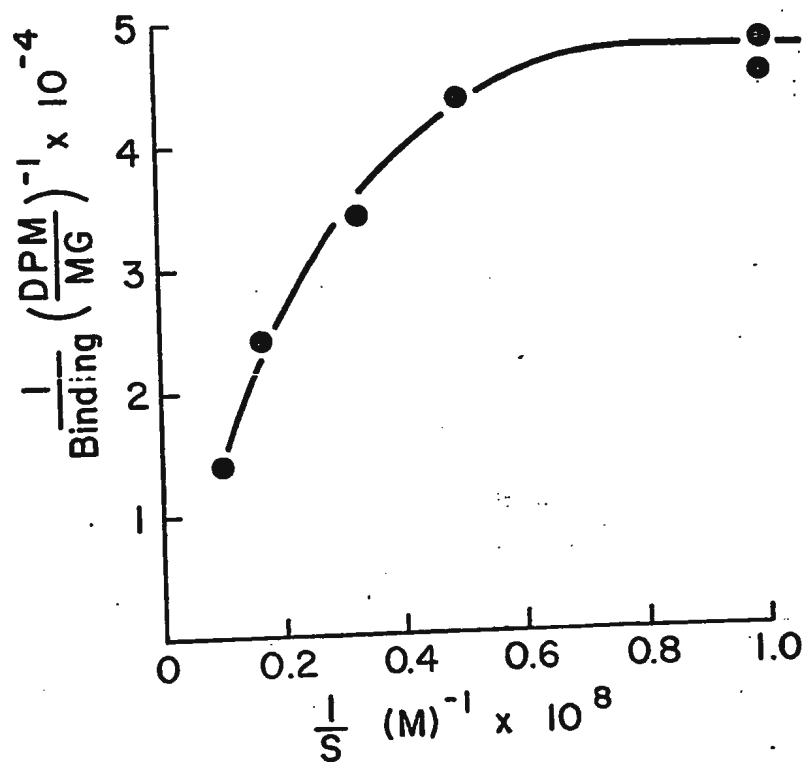


FIGURE 10

Fig. 11.

Double reciprocal plot for cytosol (aldosterone added after isolation).

$^3\text{H}$ -aldosterone was added to aliquots of isolated cytosol to give final concentrations ranging from  $10^{-8}$  to  $10^{-7}$  M. Other experimental details are as outlined in Methods. Aliquots of cytosol from the pooled kidneys of 4 rats were used to obtain each point on the graph.

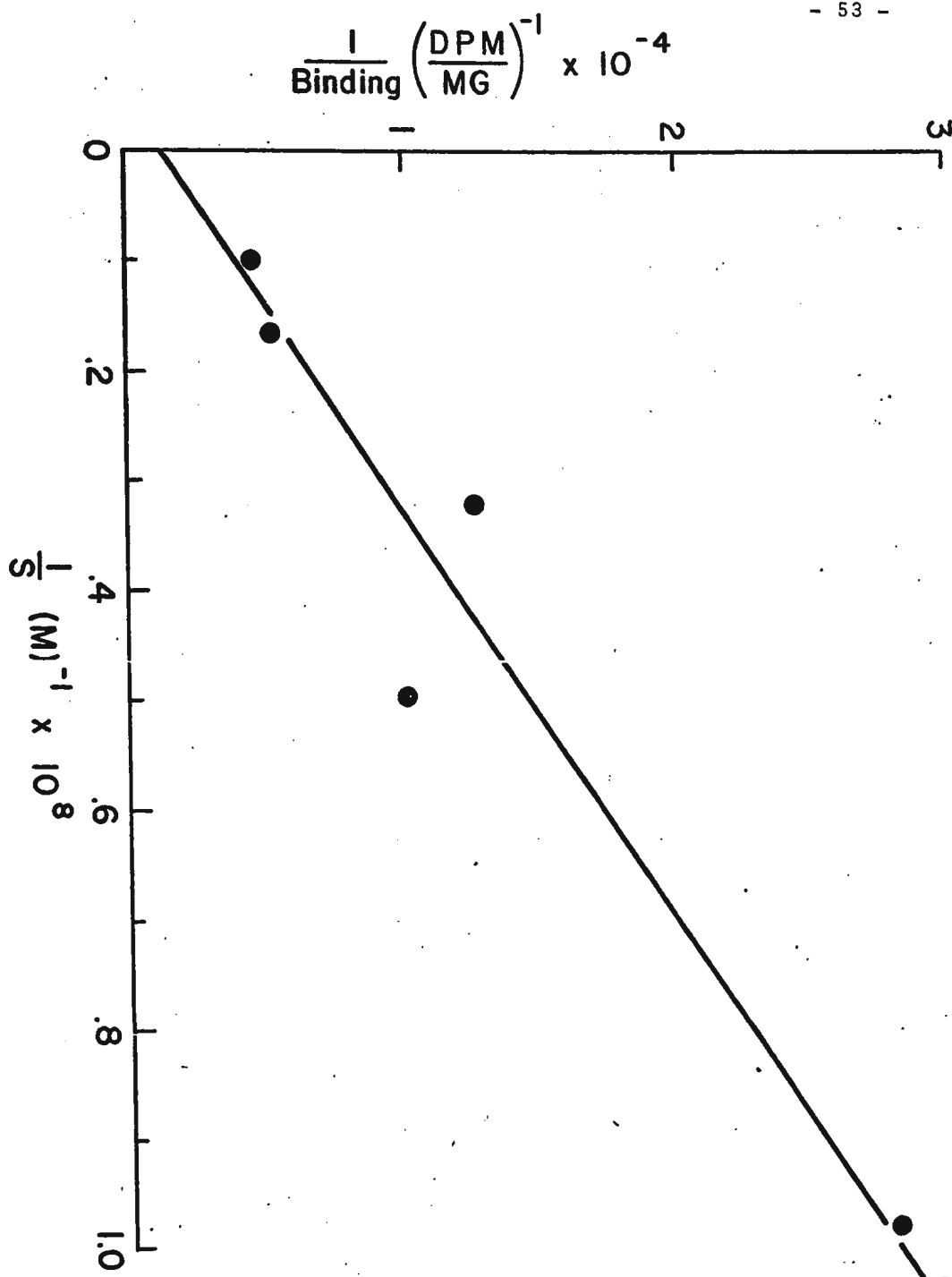


FIGURE 11

Fig. 12.

Double reciprocal plot for cytosol (aldosterone added to the isolation media).

<sup>3</sup>H-aldosterone was added to the isolation media to give final concentrations ranging from  $10^{-8}$  to  $2 \times 10^{-7}$  M. Other conditions were as outlined in Fig. 12 and in Methods.

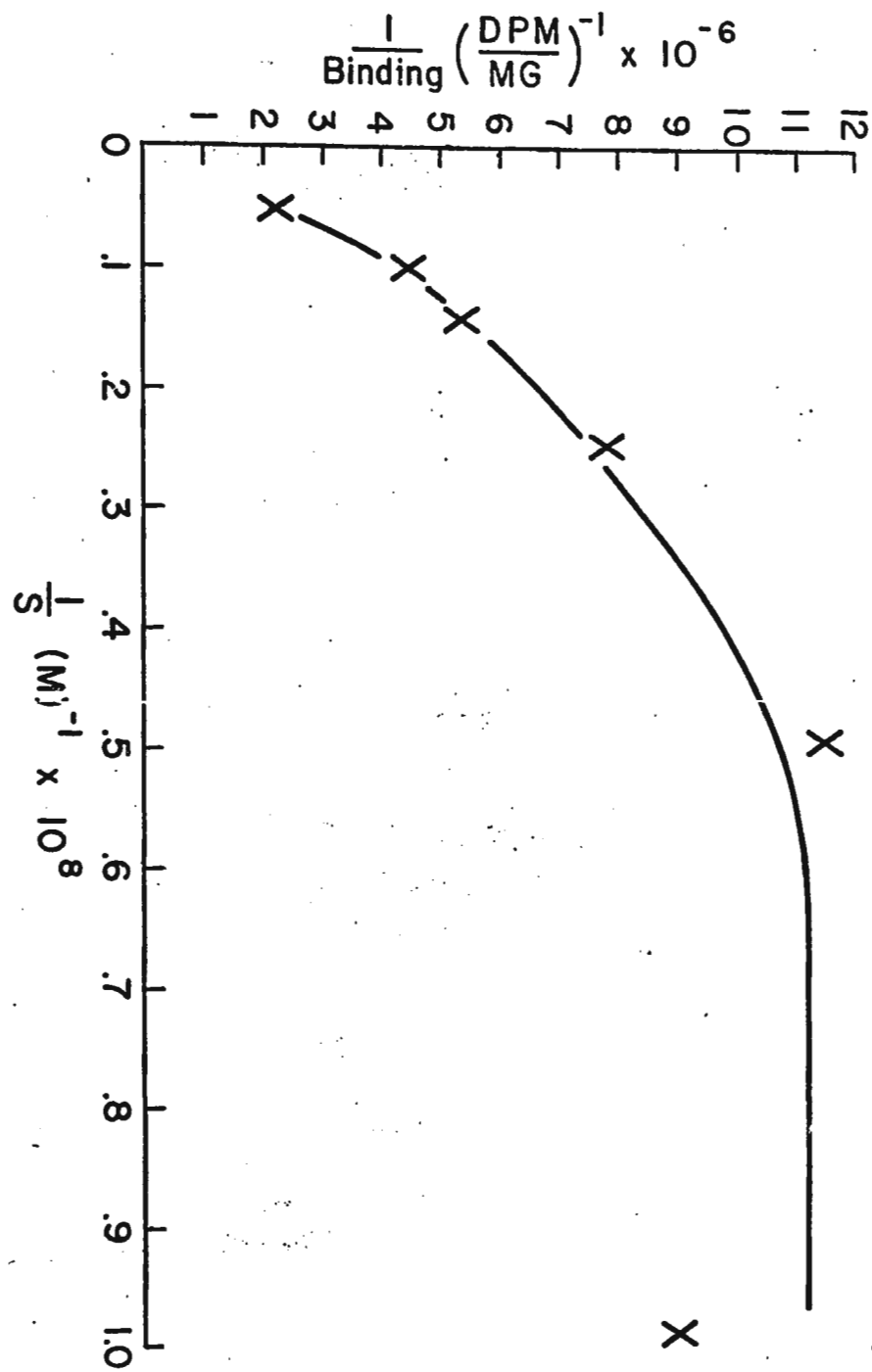


FIGURE 12

## 5. Further Experiments on Aldosterone Binding in vitro

### a. Effects of nucleotides on aldosterone binding -

c-AMP has been implicated as a mediator in the action of some hormones<sup>34,35</sup> and recently Vonderhaar, Kim, and Mueller have reported that soluble estrogen receptors of rat uteri may be modified by imidazole compounds including ATP and c-AMP<sup>91</sup>. The effects of ATP and c-AMP on the binding of aldosterone to receptors in nuclear extracts and cytosol fractions of rat kidney are shown in Table X. ATP has little effect on the binding of aldosterone by nuclear or cytoplasmic receptors and c-AMP has little effect on binding in nuclear extracts. However, c-AMP appears to inhibit the binding of aldosterone to components in the cytosol fraction. This offers additional evidence that the aldosterone binding components of nuclear extracts and of cytosol may be different.

### b. Aldosterone binding in heart and liver -

The possibility that aldosterone may be bound by components in tissues other than kidney was examined. The results of these studies are presented in Table XI. Aldosterone-binding components are present in both nuclear extracts and cytosol fractions of liver, with specific activities averaging 50 and 60 percent those of the respective kidney fractions. Specific activities of nuclear extracts and cytosol fractions of heart average 7 and 25 percent of those of kidney fractions. The higher specific activities



TABLE X

EFFECTS OF NUCLEOTIDES ON ALDOSTERONE BINDING BY KIDNEY COMPONENTS.

In each experiment, one kidney from each of three rats were combined and used as the control; the remaining 3 kidneys served as the test.  $^3\text{H}$ -aldosterone (final concentration  $5 \times 10^{-10}\text{M}$ ) was added to the isolation media of controls.  $^3\text{H}$ -aldosterone and the indicated nucleotide were added to the isolation media of tests. Incubation for 2 1/2 hr at  $0-4^\circ$  was carried out before assay. Results are averages of two experiments.

<u>Nucleotide</u>	<u>Specific Activity</u>	
	<u>dpm/mg protein</u>	
	<u>N.E.</u>	<u>Cytosol</u>
None (control)	1051	1800
ATP (0.1 mM)	1175	1814
None (control)	2358	3075
c-AMP (0.1 mM)	2281	2125

TABLE XI

ALDOSTERONE BINDING IN HEART AND LIVER.

<sup>3</sup>H-aldosterone (final concentration  $5 \times 10^{-10}$  M) was added after isolation of the fractions. Isolation of nuclear extracts and cytosol fractions, and estimation of radioactivity and protein were as described for kidney in Methods. The values shown are the result of pooling the respective organs of 3 rats.

	<u>Specific Activity</u> (dpm/mg protein)		<u>Percent Activity</u> *	
	<u>N.E.</u>	<u>Cytosol</u>	<u>N.E.</u>	<u>Cytosol</u>
Kidney	1000	609	100	100
Liver	491	373	49	61
Heart	71	149	7	25

\* The activities of kidney fractions were taken as 100%.

of liver fractions may be a result of non-specific binding due to the presence of steroid-metabolizing enzymes in the liver.

c. Aldosterone binding in male and female animals -

Female rats were used throughout this work, except where male rats are specified. Edelman used male rats in his studies<sup>54,72</sup>. In Table XII we demonstrate that in the binding of aldosterone to kidney components, very little variation in specific activities may be attributed to sex differences. In fact, our previous results indicate that variation in individual rats of the same sex may produce results which show greater differences than those shown in Table XII. It is concluded that no distinguishable differences are apparent in the concentrations of aldosterone-binding proteins in kidneys from male and female animals.

d. Aldosterone binding by plasma proteins -

The possibility that a binding protein with a high affinity for aldosterone may be present in plasma was investigated. A sample of blood was collected from the dorsal aorta of an adrenalectomized rat. A nuclear extract (of erythrocytes) and a cytosol fraction (blood serum) were obtained with <sup>3</sup>H-aldosterone present in the isolation media. Incubation of nuclear extract and cytosol for 2 1/2 hours at 0° yielded complexes with specific activities of 207 and 432 DPM/mg. protein respectively. These values are much lower than those obtained

TABLE XII

ALDOSTERONE-BINDING BY KIDNEY COMPONENTS OF MALE AND  
FEMALE RATS.

<sup>3</sup>H-aldosterone (final concentration  $5 \times 10^{-10}$ M) was included in the isolation media of all experiments, and the values shown indicate averages of 3 experiments each of which employed the pooled kidneys of 2 rats. All animals had been adrenal-ectomized 6 days prior to sacrifice.

	<u>Specific Activity</u> <u>(dpm/mg protein)</u>	
	<u>N.E.</u>	<u>Cytosol</u>
Males	1316	1691
Females	1291	1865

routinely for kidney fractions when aldosterone is present in the isolation media; hence, little of the kidney binding can be attributed to serum contamination.

e. Sephadex G-25 concentration of nuclear extract and cytosol -

Previously in this work we presented evidence that binding in nuclear extracts may be partially dependent on protein concentration (See Section 2.d). To reinforce this view, we concentrated the fractions using Sephadex G-25 according to the method of Flodin, Gelotte, and Porath<sup>92</sup>. However, the specific activities obtained were lower than the controls. It is possible that an increased rate of dissociation of complexes occurs in the presence of dry Sephadex and hence the method is not applicable to our work.

6. Attempts at Purification of Aldosterone-Macromolecular Complexes

Obviously, the ideal system for studying hormone receptors in vitro is the pure receptor which has been separated from contaminating components. Unfortunately, attempts at purification of the aldosterone receptor of rat kidney reveal that, in our hands, such an ideal system does not appear to be easily obtained.

a. Purification on Sephadex G-200 -

Since aldosterone-binding proteins are excluded from Sephadex G-50 and G-75 gels and since our results suggest heterogeneity of aldosterone-binding proteins, we attempted to fractionate the complexes on Sephadex G-200 at 0°.

When  $^3\text{H}$ -aldosterone was included in the isolation media, fractionation of the cytosol on Sephadex G-200 revealed only one diffuse peak of radioactivity which coincided with the elution volume of free aldosterone. The elution pattern suggested that the aldosterone was interacting with the Sephadex gel and aldosterone-macromolecular complex dissociation was probably occurring on the column.

When complexes were formed after isolation of fractions, a similar elution pattern was obtained.

Chromatography of the cytosol on Sephadex G-200 when a 10-fold concentration (i.e.  $5 \times 10^{-9}\text{ M}$ ) of  $^3\text{H}$ -aldosterone were present in the isolation media produced similar results.

Equilibrating the eluting buffer with  $^3\text{H}$ -aldosterone ( $5 \times 10^{-10}\text{ M}$ ) did not succeed in saturating the aldosterone binding sites on the gel and aldosterone again was removed from the column in one large diffuse band.

We conclude that aldosterone probably interacts with Sephadex G-200 and further attempts to fractionate complexes on G-200 were abandoned.

b. Purification by sucrose density gradient centrifugation -

Gorski's group has employed sucrose density gradients successfully to study estrogen-binding proteins of rat uterus. Similarities in the properties of estrogen-binding proteins of rat uterus and mineralocorticoid-binding proteins of rat kidney encouraged us to attempt analysis and possible purification of aldosterone-binding proteins by this method.

Analysis of both nuclear extracts and cytosol fractions on 15-30 percent sucrose density gradients revealed only one diffuse band of radioactivity near the top of the gradient. No bound aldosterone could be distinguished from free aldosterone. It was concluded that aldosterone-macromolecular complexes probably dissociated in the sucrose medium during centrifugation and hence no satisfactory results could be obtained by this method.

## DISCUSSION

### Importance of the Aldosterone-binding System

The available evidence suggests that aldosterone-binding components are important in the mechanism of action of aldosterone and related mineralocorticoids. The supporting evidence for this statement may be summarized briefly:

- a) Mineralocorticoids regulate active sodium transport across epithelial cells of certain tissues by a process which involves induction de novo of protein synthesis<sup>17</sup>, initiated by stimulation of DNA-dependent RNA synthesis<sup>18,20</sup>.
- b) <sup>3</sup>H-aldosterone was found to be preferentially distributed over the nuclei of toad bladder epithelial cells while <sup>3</sup>H-progesterone was randomly distributed<sup>17</sup>.
- c) <sup>3</sup>H-aldosterone-macromolecular complexes were isolated from the nuclear and cytosol fractions of rat kidney after in vivo injection of hormone, and these macromolecules were identified as proteins by several criteria<sup>40</sup>.
- d) In an in vitro system, spiro lactone inhibited the formation of <sup>3</sup>H-aldosterone complexes at concentration ratios that inhibit the action of aldosterone in the rat in vivo<sup>40</sup>.

The way in which these receptors may be involved in the action of aldosterone is outlined in Fig. 13. We decided to ask the following questions:

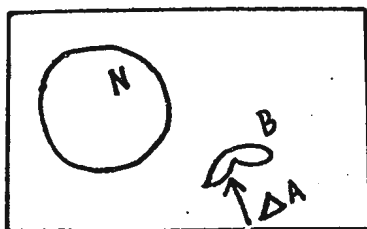
- a) What are the properties of aldosterone-binding proteins?



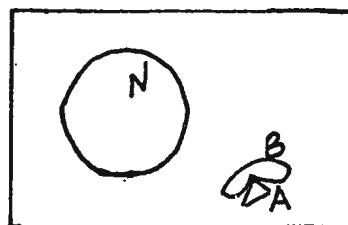
Fig. 13. Hypothetical Model for Mode of Action of Aldosterone.

The rectangle represents a kidney epithelial cell.

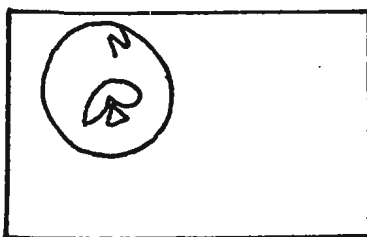
- N - nucleus
- B - aldosterone-binding protein
- A - aldosterone
- C - chromatin
- RNA - the aldosterone-specific RNA
- AA - amino acids
- Rbs - ribosomes
- P - aldosterone-specific protein



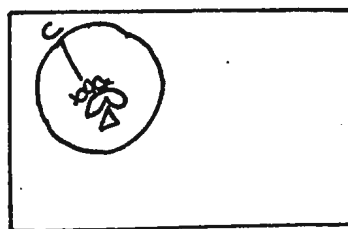
1. Aldosterone enters the cell.



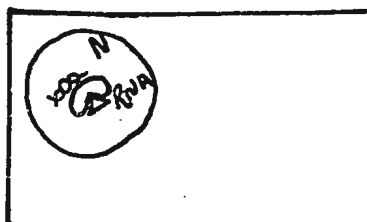
2. Aldosterone binds to a cytoplasmic protein.



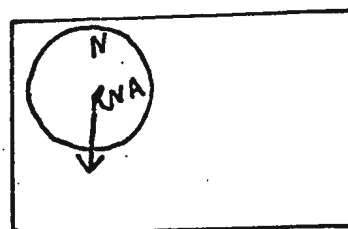
3. The complex moves to the nucleus.



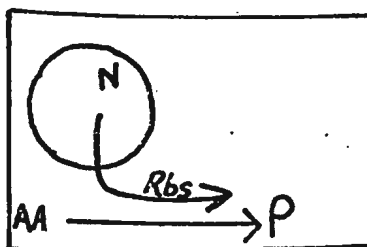
4. The complex attaches to chromatin.



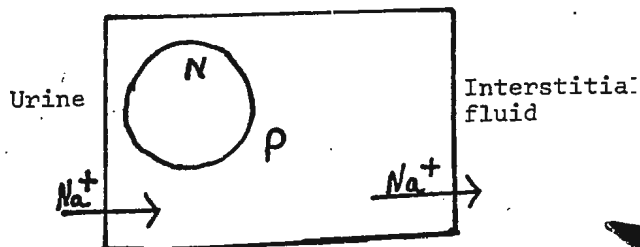
5. Aldosterone-specific RNA synthesis occurs.



6. The newly synthesized RNA leaves the nucleus.



7. Synthesis of aldosterone-specific protein occurs.



8. Sodium retention is increased.

FIGURE 13

- b) Is there more than one aldosterone-binding protein in rat kidney?
- c) What is the specificity of the binding components?
- d) How great is their affinity for aldosterone?

#### Stability of Aldosterone-binding components

Our results confirm that aldosterone binds in vivo and in vitro to macromolecular components of the nuclear and cytosol fractions of rat kidney, and that the complexes formed are unstable (See Figs. 3, 7, and 8). Their stability, however, can be enhanced in several ways:

1) Storage in the nucleus as aldosterone-macromolecular complexes. The increased stability afforded to the complexes under these conditions may be partially explained by the dependence of complexes on protein concentration as shown in section 2.d. In addition, Figs. 7 and 8 show that the presence of aldosterone also stabilizes the macromolecular components and radioautographs demonstrate that kidney nuclei tend to concentrate aldosterone<sup>17</sup>. This suggests a second explanation of why complexes which remain in the nucleus are protected from dissociation.

2) The stabilizing effect of 20% glycerol as depicted in Fig. 3 confirms results published by Herman and Edelman<sup>73</sup>. Talalay has suggested that the protective effect of various organic solvents is due to their common property of stabilizing networks of "structured" water molecules which are essential to the maintenance of the proper spatial configuration of the protein in the native state<sup>93</sup>. Such suggestions have not

as yet been proven and the mechanism of the stability effect of glycerol remains obscure.

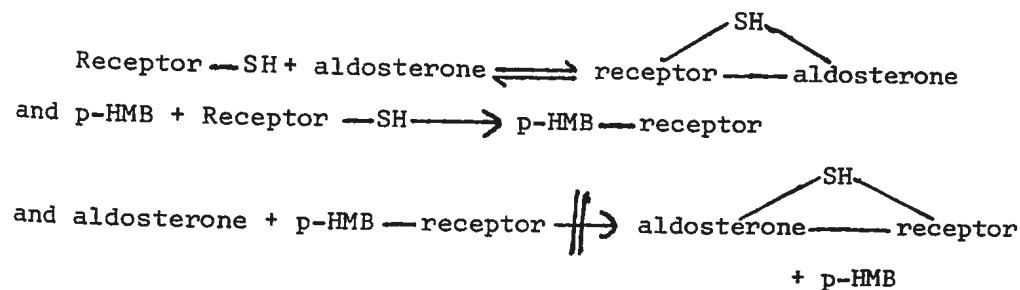
These studies on the stability of aldosterone-macromolecular complexes support Edelman's view that the binding components are proteins. The stabilization of complexes by the presence of aldosterone is analogous to the protection of enzymes from denaturation by the presence of specific substrates, and glycerol has been shown to have a stabilizing effect on some enzymes<sup>94,95</sup>.

#### Other Properties of Aldosterone-binding Components

Fig. 4 shows that aldosterone-macromolecular complexes formed in vitro are dissociated by p-HMB. This sulfhydryl reagent, which inactivates some enzymes by interacting with essential -SH groups on the molecule, has previously been shown to dissociate aldosterone-macromolecular complexes formed in vivo<sup>40</sup> and 17 $\beta$ -estradiol-protein complexes isolated from rat uterus<sup>62</sup>. By analogy with these systems, the protein nature of the in vitro aldosterone-binding system is suggested.

Table II indicates that the presence of relatively low concentrations of aldosterone causes little change in the amount of complex dissociation by p-HMB. If the aldosterone binding site is remote from the p-HMB binding groups, the reduced binding of aldosterone may be the result of a conformational change which is unfavourable to the binding of the hormone. If

an -SH group is directly involved in the binding site for aldosterone, an existing equilibrium between free and bound aldosterone would rapidly favor the free hormone as many of the -SH groups were excluded by their reaction with p-HMB; i.e. if we have an equilibrium,



then aldosterone protection can only occur at saturating aldosterone concentrations.

The c-AMP inhibition of aldosterone binding in cytosol as shown in Table X can occur if c-AMP acts as a competitive inhibitor of aldosterone or if the nucleotide can affect the binding moiety in such a way that its ability to bind aldosterone is impaired. The latter explanation seems the more probable one. Although a receptor protein for c-AMP has been reported in adrenal cortical and other tissues<sup>44,45</sup>, it seems unlikely that the two binding proteins are identical. In addition, other workers<sup>91</sup> have shown recently that various nucleotides, including c-AMP, have a stimulatory affect on the binding of 17  $\beta$ -estradiol to receptors in rat uteri. Hence, the action of various nucleotides in stimulating or inhibiting the binding of steroids to macromolecules may be an allosteric one as suggested

by Monod et al for the action of small molecules in activating or inactivating certain enzymes<sup>96</sup>.

A rapid increase in specific activities (expressed as dpm per mg protein) of aldosterone-macromolecular complexes in both nuclear extracts and cytosol fractions of rat kidney occurs after adrenalectomy, as is seen clearly in Figs. 5 and 6. This phenomenon may occur as a result of the rapid metabolism and disappearance of endogenous aldosterone and other adrenocorticoids after adrenalectomy. Our results show that other exogenous steroids inhibit the formation of aldosterone-macromolecular complexes in vitro, which suggests that such steroids compete with the radioactive aldosterone for sites on the binding molecule(s). Alternately, an increase, specifically in the amount of aldosterone-binding protein, after adrenalectomy, would account for the observed increase in specific activities. Although an increased amino acid incorporation into protein by kidney microsomes of adrenalectomized rats has been reported at times ranging from 3 hours to 31 days after the operation<sup>82</sup>, it is doubtful that the rate of production of aldosterone binding protein is specifically increased (See Figs. 5 and 6).

#### Distribution of Aldosterone-binding Components

##### a. Subcellular distribution in kidney -

A two-step mechanism involving binding of hormone to cytoplasmic receptor and subsequent movement of the hormone-receptor complex from the cytoplasm to the nucleus has been

suggested for estradiol<sup>36,52</sup> (c.f. Fig. 13). Table III shows that a higher nuclear extract to cytosol binding ratio is obtained in vivo than in vitro. This is compatible with a two-step mechanism for aldosterone (see Fig. 13) since by this mechanism <sup>3</sup>H-aldosterone enters the cell after injection, binds to its receptor in the cytoplasm, is transferred as an aldosterone-receptor complex to the nucleus where binding to chromatin takes place and subsequently, the action of aldosterone on sodium retention is expressed. In in vitro experiments, complexes formed in the cytoplasm cannot move to the nucleus — hence a lower nuclear extract to cytosol binding ratio is expected.

Table IV of our results shows, however, that an increased protein concentration, caused by the addition of bovine serum albumin, enhances aldosterone binding to receptors in nuclear extracts. This finding suggests that the higher binding ratio obtained in vivo may be due to the higher nuclear protein concentration compared to that of nuclear extracts and may be interpreted to argue against a two-step mechanism for aldosterone. However, a recent paper by Swaneck et al<sup>72</sup> acknowledges a personal communication from Goodman and Edelman in which these authors claim to have evidence that the cytoplasmic aldosterone-protein complex is formed first, followed by formation of a soluble nuclear aldosterone-binding protein complex and finally by a chromatin-aldosterone-binding protein complex. Such a situation would be compatible with a two-step

mechanism for aldosterone action, but thus far, no publication of these results has appeared.

b. Distribution in other tissues -

It is interesting that liver and heart tissues appear to contain small amounts of macromolecular components which are capable of binding aldosterone in vitro. Although no attempt was made to determine the specificity of these binding moieties, the presence of such components in tissues other than kidney appears significant because it is becoming increasingly apparent that few, if any, hormones have a single target tissue, and if receptors are functional in mechanism of action, they would be expected in all target tissues. Recent reports have appeared which suggest that aldosterone may exert its effect in erythrocytes<sup>97</sup> and in skeletal muscle<sup>98</sup>. Also noteworthy is a recent report that estradiol receptors have been found in rabbit hypothalamus tissue<sup>99</sup>. Our results are in agreement with those obtained by Swaneck et al<sup>54</sup>. These workers found that aldosterone binds in vivo to liver components and report that aldosterone binds also to components in duodenal mucosa, spleen, and brain.

Physiological Significance of Aldosterone-binding Components

It appears that aldosterone-binding components have a physiological function in the mechanism of action of the hormone. The increased specific activities of complexes of adrenalectomized rats compared to controls, as evidenced in Table V, suggests that the receptor normally binds endogenous hormone. Other workers<sup>40</sup> have shown that unlabelled aldosterone



competes with  $^3\text{H}$ -aldosterone for the receptor in vitro and, in addition, that the steroid specificities for the aldosterone binding site are the same in the nuclear and cytosol fractions both in vivo and in vitro and are in proportion to the mineralocorticoid or inhibitor potencies of these steroids. Similarly, a physiological role for estrogen receptors is suggested since the binding of estrogen to protein is inhibited by steroid and non-steroid estrogens but not by non-estrogen steroids<sup>47</sup>.

The exact role of steroid-binding proteins in the physiological action of these hormones has not as yet been established. Talwar and coworkers have reported that estrogen-protein complexes formed in the rat uterus may be involved in the regulation of RNA polymerase activity<sup>51</sup>. Dahmus and Bonner have proposed that the primary action of cortisol involves derepression at the level of DNA transcription<sup>22</sup>. A direct evaluation of the role of steroid-binding proteins in the mechanism of hormone action, however, requires purification of these proteins and characterization of their physical and chemical properties. Our attempts at purification of the aldosterone-macromolecular complexes on Sephadex G-200 and on sucrose density gradients have not been successful. The failure to purify complexes by these methods may probably be attributed to the tendency of aldosterone-macromolecular complexes to dissociate during the purification procedure.

### Heterogeneity of Aldosterone-binding Components

The heterogeneity of estrogen receptors of rat uteri has been well established<sup>48,49,61-64</sup> and some evidence has appeared which suggests that aldosterone-binding components in rat kidney and in the urinary bladder of the toad may be heterogeneous<sup>70,71</sup>. Our results strongly support the view that aldosterone-binding components of rat kidney are heterogeneous.

The increase in specific activities of complexes of both nuclear extracts and cytosol fractions when aldosterone is present in the isolation media indicates that either the presence of aldosterone stabilizes the aldosterone-receptor complex or that additional components bind aldosterone when the hormone is present from the start of homogenization. The results presented in Table VII favour the latter explanation. The amount of inhibition by 17 $\beta$ -estradiol is not appreciable when hormones are added after isolation of fractions. This confirms results of other workers<sup>40</sup>. However, the inhibition of binding by 17 $\beta$ -estradiol becomes significant in both fractions when hormones are present in the isolation media. Such results are compatible with the hypothesis that there are at least two different aldosterone-binding components in rat kidney, one of which is highly unstable and relatively non-specific. This hypothesis is further strengthened when the comparison (shown in Tables VIII and IX) of inhibition of

binding by other steroids in pre-isolated fractions and when steroids are included in the isolation media, is considered.

The time course study shows that under all the conditions described, dissociation of aldosterone-protein complexes occurs with time. If the fractions are prepared in the absence of aldosterone, there is an initial rapid loss of binding ability followed by a slower rate of decay. Addition of aldosterone to pre-isolated fractions, immediately after isolation, gives considerable protection; but addition of hormone to the homogenate after homogenization does not give higher binding than if it is added after fractionation. The hormone must be present at the start of homogenization and throughout the isolation procedure for maximum protection. These results are again compatible with our proposal of heterogeneity of aldosterone-binding proteins.

The double reciprocal plots for binding vs aldosterone concentration, presented in Figs. 9 - 12, are interesting. It seems that in both nuclear extract and cytosol, straight line plots are obtained when hormone is added after isolation of fractions. On the other hand, such plots do not yield straight lines when aldosterone is present in the isolation media. Such results imply either multiple binding proteins for aldosterone or binding proteins whose ability to express cooperative interactions is unstable. The simplest interpretation is heterogeneity. These in vitro results confirm the suggestions of heterogeneity made by other workers using in vivo systems<sup>70,71</sup> and in the light of other evidence discussed above, the case

for multiple binding proteins for aldosterone appears strong.

However, the apparent dissociation constants derived from the straight line double reciprocal plots pose problems. It would be expected that the physiologically active binding protein for aldosterone would have an affinity for aldosterone such that its dissociation constant would be of the same order of magnitude as the concentration of aldosterone in kidney. If one assumes that the physiological concentration of aldosterone in kidney approximates that of plasma, then the calculated dissociation constants for aldosterone binding proteins or rat kidney appear to be one or two orders of magnitude too high. Hence it appears that either the stable components under consideration here are not the true physiologically active aldosterone-binding proteins, or that aldosterone concentrations in kidney are much higher than plasma concentrations. Although Edelman's group has claimed that the aldosterone-binding proteins studied in vitro are mineralocorticoid specific, results of studies on inhibition by other steroids as shown in Table IX indicate that some non-specific binding occurs.

In a recent study it was shown that the supernatant fraction of human uterus contained estrogen receptors that could be separated from the free hormone by Sephadex chromatography<sup>100</sup>. The author reports that there are at least two binding sites for estrogen as determined from Scatchard plots; that pre-incubation with unlabelled hormone, estrogen antagonists, or

sulfhydryl reagents strongly inhibits the binding of  $^3\text{H}$ -estradiol; and that pH and temperature optima for in vitro binding are apparent. This study reveals many similarities in the properties of receptors in human uterus and those of rat uterus, and many of the results are analogous to results obtained in this work on aldosterone-binding components. One is tempted to extrapolate the results obtained here for aldosterone-binding proteins of rat kidney to the human system, but such an extrapolation may prove difficult to ascertain since suitable material necessary for such a study would be most difficult to obtain.

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# APPENDIX

Derivation of a double reciprocal equation for aldosterone binding -

Aldosterone apparently combines with a binding moiety to form a complex according to the equation



P = binding protein

AP = aldosterone-protein complex.

Hence by the law of mass action,

$$\frac{[A][P]}{[AP]} = K \quad \dots (1) \quad \text{where } K \text{ is a constant}$$

[A]

[A] is concentration\* of aldosterone

[P] is concentration\* of protein

[AP] is concentration\* of complex

Now if  $[P_T]$  is the original concentration of binding protein before addition of  $^3\text{H}$ -aldosterone, then the concentration of free binding protein, [P], at any time after addition of aldosterone, A, will be  $[P] = [P_T] - [AP]$ .

Similarly,  $[A] = [A_T] - [AP]$  where  $A_T$  is the total aldosterone concentration (i.e. free + bound)

but  $[AP] \ll [A]$

$$\therefore [A] = [A_T]$$

Now, substituting for [P] in Equation (1) we obtain

---

\* expressed in molarity

$$[A]([P_T] - [AP]) = K_D[AP]$$

$$[A][P_T] - [A][AP] = K_D[AP]$$

$$[A][P_T] = K_D[AP] + [A][AP]$$

$$\frac{[A][P_T]}{[AP]} = K_D + [A]$$

$$\text{or } \frac{[P_T]}{[AP]} = \frac{K_D}{[A]} + 1$$

$$\text{i.e. } \frac{1}{[AP]} = \frac{K_D}{[P_T]} \cdot \frac{1}{[A]} + \frac{1}{[P_T]} \quad \dots\dots(2)$$

This is the equation of a straight line whose slope is

$$\frac{K_D}{[P_T]} \quad \text{and ordinate intercept } \frac{1}{P_T} .$$

Equation (2) is analogous to the Lineweaver-Burk equation for

$$\text{enzymes } \frac{1}{v} = \frac{K_m}{v} \cdot \frac{1}{S} + \frac{1}{v} .$$

PUBLICATIONS ARISING FROM THIS WORK -

1. Properties of Renal Aldosterone-Binding Proteins.

A.J. Mills, J.F. Wheldrake and L.A.W. Feltham, Biochem. J.  
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2. Aldosterone-Binding Proteins in Rat Kidney. A.J. Mills,  
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3. Aldosterone-Binding Proteins in Rat Kidney. A.J. Mills,  
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